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Differences in the Intestinal Microbiome and Lipidome of Dogs Diagnosed with Idiopathic Inflammatory Bowel Disease and Food-Responsive Diarrhea before and after the Induction Phase of Treatment

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Dedicated to my parents

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AIC	Akaike information criterion
AIEC	adherent-invasive *E.coli*
ANOSIM	analysis of similarities
ANOVA	analysis of variance
ARD	antibiotic-responsive diarrhea
BCS	body condition score
CBC	complete blood count
CCECAI	canine chronic enteropathy clinical activity index
CFU	colony forming units
CIE	chronic inflammatory enteropathies
cTLI	canine trypsin-like-immunoreactivity
DAMPs	damage-associated molecular patterns
DHA	docosahexaenoic acid
DI	dysbiosis index
EPA	eicosapentaenoic acid
ESI	electrospray ionization
FISH	fluorescence *in situ* hybridization
FMT	fecal microbiota transplantation
FRD	food-responsive diarrhea
GI	gastrointestinal
HDL	high-density lipoprotein
HILIC	hydrophilic interaction liquid chromatography
IBD	inflammatory bowel disease
IRD	immunosuppressant-responsive diarrhea
LDA	linear discriminant analysis
LDL	low-density lipoprotein
LEfSe	linear discriminant analysis effect size
lysoPC	lysophosphatidylcholine
MALDI	matrix-assisted laser desorption / ionization
MAMPs	microbe-associated molecular pattern molecules
NOD2	nucleotide oligomerization domain-2
OTU	operational taxonomic unit

PC	phosphatidylcholine
PCA	principal component analysis
PCoA	principal coordinates analysis
PLE	protein-losing enteropathy
PrComp	principal component
PUFA	polyunsaturated fatty acid
QIIME	quantitative insights into microbial ecology
qPCR	quantitative real-time PCR
SRD	steroid-responsive diarrhea
TLR	toll-like receptor
TRD	tylosin-responsive diarrhea
VLDL	very low-density lipoprotein
WSAVA	World Small Animal Veterinary Association
16S rRNA	16S ribosomal RNA

1 INTRODUCTION

Intestinal diseases and their corresponding clinical signs are a common reason for a dog to be presented to a veterinary hospital. Diarrhea represents one of the characteristic clinical signs of intestinal disease and can be classified as small bowel diarrhea, large bowel diarrhea, or mixed small and large bowel diarrhea (ALLENSPACH 2010a, HALL and GERMAN 2010). Small bowel diarrhea is characterized by typical clinical signs that include increased fecal volume, normal to only mildly increased frequency of defecation, melena, and in some cases steatorrhea, as well as weight loss (HALL and GERMAN 2010). Large bowel diarrhea, on the other hand, is associated with an increased frequency of defecation, tenesmus, fecal urgency, often with mucus in the stool, and in some cases also hematochezia (ALLENSPACH 2010a). However, a clear differentiation between the two forms of diarrhea is not always possible, especially because many dogs show a combination of small and large bowel diarrhea.

In addition to diarrhea, also vomiting, weight loss, poor appetite (hyporexia or anorexia), abdominal pain, borborygmus, or flatulence are common clinical signs in dogs with chronic intestinal disease (HALL and GERMAN 2010). However, none of these clinical signs are pathognomonic for intestinal disease. Therefore, a thorough patient history followed by a clinical and clinicopathological evaluation are essential in order to exclude other extragastrointestinal diseases (e.g., pancreatic disease, chronic liver disease, chronic renal disease, or several endocrine disorders) as a cause of the patient's clinical signs.

Intestinal diseases can be categorized into acute or chronic diseases (HALL and GERMAN 2010). Infectious gastroenteritides (e.g., viral, bacterial, parasitic, or fungal enteritis), the acute hemorrhagic diarrhea syndrome (formerly known as idiopathic hemorrhagic enteritis) (BURROWS 1977, UNTERER et al. 2014, MORTIER et al. 2015), self-limiting acute gastroenteritis of unknown origin, acute transit disorders (e.g., ileus), dietary indiscretion, and toxin ingestion are the main causes of acute gastrointestinal clinical signs in dogs (ALLENSPACH and GASCHEN 2011).

Chronic inflammatory enteropathies (CIE), which are characterized by persistent or recurrent clinical signs of gastrointestinal disease for at least 3 weeks' duration (GERMAN et al. 2003a, WASHABAU et al. 2010), together with intestinal neoplasia and chronic parasitic diseases, are a common cause of chronic intestinal signs in dogs. Based on the response to treatment, chronic inflammatory enteropathies are categorized into food-responsive diarrhea (FRD), antibiotic-responsive diarrhea (ARD), and idiopathic inflammatory bowel disease (IBD) (GERMAN et al. 2003a, ALLENSPACH et al. 2007, DANDRIEUX 2016, ERDMANN and HEILMANN 2017). Dogs with FRD will respond favorably to dietary modification alone

(ALLENSPACH et al. 2007, MANDIGERS et al. 2010), whereas dogs with ARD require the use of an antibiotic for clinical signs to resolve (GERMAN et al. 2003a, WESTERMARCK et al. 2005, KILPINEN et al. 2011). IBD is defined by the presence of chronic gastrointestinal signs, histologic evidence of intestinal inflammation, and the need for anti-inflammatory and / or immunosuppressive treatment (GERMAN et al. 2003a, DAY et al. 2008, WASHABAU et al. 2010, ALLENSPACH et al. 2016).

Until today, the exact pathogenesis of IBD in companion animals remains elusive. Recent studies support the hypothesis that a combination of a genetic predisposition (BURGENER et al. 2008, KATHRANI et al. 2010, ALLENSPACH et al. 2010b, KATHRANI et al. 2011, KATHRANI et al. 2012, KATHRANI et al. 2014), dietary and environmental factors, and the intestinal microbiota can contribute to the development of IBD in dogs (SIMPSON and JERGENS 2011, HEILMANN and ALLENSPACH 2017). Recently, the role of the intestinal microbiota in particular has attracted a large amount of attention and intestinal or fecal dysbiosis has been demonstrated in dogs with IBD, reflected mainly by an increase in Proteobacteria and a decrease in *Faecalibacterium* spp. compared to healthy dogs (SUCHODOLSKI et al. 2010, SUCHODOLSKI et al. 2012a, HONNEFFER et al. 2014, MINAMOTO et al. 2015, CASSMANN et al. 2016).

To the author's knowledge, there are no studies that evaluate the possibility of differences in the intestinal microbiota in dogs diagnosed with IBD and dogs with FRD. Therefore, the first objective of this study was to compare the duodenal and colonic mucosal microbiota between dogs with IBD and dogs diagnosed with FRD. Further, the intestinal mucosal microbiota in each dog was evaluated before and after the induction phase of treatment to determine the possibility of an effect of treatment on the intestinal microbial composition.

In addition to the further understanding of the role of microbial changes in canine patients with IBD, the search for potential novel treatment options that can modulate the intestinal microbiome is of particular clinical importance. The use of probiotics in canine IBD has become increasingly popular, and the use of probiotic mixtures in particular has shown promising results (SAUTER et al. 2005, ROSSI et al. 2014). Polyunsaturated fatty acids (PUFAs) were demonstrated to have immunomodulatory and anti-inflammatory properties both in humans (SIMOPOULOS 2002, CALDER 2013, UNGARO et al. 2017) and in veterinary patients (MUELLER et al. 2004, ABBA et al. 2005, ONTSOUKA et al. 2010, BAUER 2011, ONTSOUKA et al. 2012), and are commonly used in the treatment of canine dermatologic diseases, such as atopic dermatitis (MUELLER et al. 2004, ABBA et al. 2005). Despite the beneficial effects seen in nongastrointestinal diseases, there are only few studies investigating the effect of dietary supplementation of PUFAs in dogs with CIE. Preliminary studies in dogs with chronic inflammatory enteropathies suggest that feeding supplemental

PUFAs can improve cholesterol homeostasis (ONTSOUKA et al. 2010) and can modulate the expression of genes regulating fatty acid uptake in the duodenum (ONTSOUKA et al. 2012). In order to shed more light on this aspect of CIE pathogenesis, the second objective of our study was to assess the systemic phospholipid profile (i.e., the circulating lipidome) of dogs with FRD or IBD both before and after treatment including supplemental PUFAs.

2 REVIEW OF LITERATURE

2.1 Chronic Inflammatory Enteropathies in Dogs

2.1.1 Definition

2.1.1.1 Diagnostic Criteria and Disease Classification

Chronic inflammatory enteropathies (CIE) are characterized by persistent or recurrent clinical signs of gastrointestinal disease, such as diarrhea, vomiting, inappetence, weight loss, abdominal pain, borborygmus or flatulence, for more than 3 weeks (GERMAN et al. 2003a, SIMPSON and JERGENS 2011, DANDRIEUX 2016, ERDMANN and HEILMANN 2017). A diagnosis of CIE also requires that no other underlying extraintestinal (e.g., pancreatic, hepatic, renal, or endocrine disorders) or intestinal diseases (e.g., endoparasites, infectious disease, motility disorders, or intestinal neoplasia) has been identified (SIMPSON and JERGENS 2011, DANDRIEUX 2016). The third criterion is that there is histologic evidence of intestinal inflammation, which is one of the cornerstones of CIE diagnosis and also further classification. Based on the quality of the inflammatory infiltrate and the predominant population of inflammatory cells, CIEs are histologically classified as either lymphoplasmacytic, eosinophilic, or mixed inflammation (GERMAN et al. 2003a, DAY et al. 2008, WASHABAU et al. 2010).

CIE can further be classified based on the patient's response to treatment as either food-responsive diarrhea (FRD), antibiotic-responsive diarrhea (ARD), or idiopathic inflammatory bowel disease (IBD) (GERMAN et al. 2003a, ALLENSPACH et al. 2007, DANDRIEUX 2016, ERDMANN and HEILMANN 2017). In dogs with FRD, clinical signs resolve after dietary modification alone (ALLENSPACH et al. 2007, MANDIGERS et al. 2010), whereas dogs with ARD show a long-lasting response to dietary management and the use of an antibiotic, for example metronidazole or tylosin (GERMAN et al. 2003a, WESTERMARCK et al. 2005, KILPINEN et al. 2011). IBD is characterized by chronic gastrointestinal signs of undetermined etiology, histologic evidence of intestinal inflammation, failure to achieve clinical remission with an elimination diet and / or antibiotic trial, and the need for anti-inflammatory and / or immunosuppressive treatment (GERMAN et al. 2003a, DAY et al. 2008, WASHABAU et al. 2010). Thus, IBD is also referred to as immunosuppressant-responsive (IRD) or steroid-responsive diarrhea (SRD) (ALLENSPACH et al. 2016, DANDRIEUX 2016), though complete remission is not achieved in all dogs with IRD / SRD.

Several dog breeds appear to be predisposed to the development of CIE. These include German shepherd dogs, which are overrepresented among dogs diagnosed with ARD (BATT et al. 1991, GERMAN et al. 2003a, ALLENSPACH et al. 2010b), Irish Setters with gluten-sensitive enteropathy (GARDEN et al. 2000), and Soft Coated Wheaten Terriers with protein-

losing enteropathy (PLE) and / or protein-losing nephropathy (LITTMAN et al. 2000). Such breed predilections raise the suspicion of genetic factors contributing to the development of CIE, though an association with genetic defects or mutations has to date not been identified.

2.1.1.2 Clinical Disease Activity

Dogs with CIE can present with a variety of clinical signs and disease severities depending on the affected site or sites and the degree of inflammation within the gastrointestinal tract (JERGENS et al. 1992, JERGENS et al. 2003, CRAVEN et al. 2004, ALLENSPACH et al. 2007, DANDRIEUX 2016). Frequent clinical signs are vomiting, diarrhea, inappetence, abdominal pain and weight loss (GERMAN et al. 2003a, DANDRIEUX 2016). These variable clinical signs and disease severities make an objective patient assessment and accurate evaluation of the response to treatment difficult. Therefore, two scoring indices have been developed in order to assess the clinical degree of illness, which is referred to as clinical disease activity, and aid in the management of clinical patients:

- canine inflammatory bowel disease activity index (CIBDAI) (JERGENS et al. 2003)
- canine chronic enteropathy clinical activity index (CCECAI) (ALLENSPACH et al. 2007).

Both clinical scoring systems include the criteria attitude / activity, appetite, vomiting, stool consistency, stool frequency, and weight loss. The CCECAI additionally includes the serum albumin concentration, presence of ascites / peripheral edema, and pruritus (ALLENSPACH et al. 2007). All criteria are scored on a scale of 0 - 3 according to the degree of alteration from normal in a specific patient (e.g., appetite: 0 = normal, 1 = slightly decreased, 2 = moderately decreased, 3 = severely decreased) (JERGENS et al. 2003, ALLENSPACH et al. 2007). The scores for each individual criterion add up to the total activity index score. Based on this score, the severity of disease is defined as clinically insignificant (score 0 - 3), mild (score 4 - 5), moderate (score 6 - 8), severe (CIBDAI score > 9; CCECAI score 9 - 11) (JERGENS et al. 2003, ALLENSPACH et al. 2007), or very severe disease (CCECAI score ≥ 12) (ALLENSPACH et al. 2007). A high clinical activity index has been associated with a negative outcome (ALLENSPACH et al. 2007).

On the basis of these clinical activity indices a patient's disease status should be regularly assessed in order to evaluate the response to treatment and guide further treatment decisions. Clinical response to treatment is reflected by the change in the activity index score where full remission is defined as a change in the total score of > 75%, partial response as a change between 25 - 75%, and a lack of response if the change in the total score is < 25%

(JERGENS et al. 2010, HEILMANN et al. 2016). Thus, the clinical activity index scores and the overall treatment response can aid in the prognostication of canine CIE.

2.1.1.3 Clinicopathologic Findings

There are no pathognomonic findings on the complete blood count (CBC) or the biochemistry profile in dogs with CIE (HALL and GERMAN 2010b). However, a CBC and biochemistry panel should always be performed in order to exclude extraintestinal diseases (HALL and GERMAN 2010). Moreover, albeit not pathognomonic, several hematologic and biochemical abnormalities have been reported in dogs with CIE (CRAVEN et al. 2004, ALLENSPACH et al. 2007, DANDRIEUX 2016). One retrospective study on canine inflammatory bowel disease revealed anemia in 12%, neutrophilia in 7%, and eosinophilia in 4% of dogs (CRAVEN et al. 2004), whereat anemia may be the result of chronic blood loss or chronic inflammation. Dogs with intestinal protein loss, especially albumin, are categorized as protein-losing enteropathy (PLE) (HALL and GERMAN 2010, DANDRIEUX 2016). Hypoalbuminemia has been identified as a risk factor for a negative outcome (CRAVEN et al. 2004, ALLENSPACH et al. 2007) leaving patients with PLE with a more guarded prognosis. The vitamins folate and cobalamin are absorbed in the intestine, with cobalamin being absorbed in the ileum. Thus, intestinal inflammation can result in decreased absorption and a subsequent deficiency of these vitamins (HALL and GERMAN 2010). Hypocobalaminemia is of particular importance, as hypocobalaminemia at the time of CIE diagnosis is also associated with a negative outcome even after a correction of the serum cobalamin deficiency through cobalamin supplementation (ALLENSPACH et al. 2007). Additionally, low serum concentrations of vitamin D at the time of diagnosis have been shown to be a negative prognostic factor for dogs with CIE (TITMARSH et al. 2015). However, until now there is no information on how vitamin D supplementation affects vitamin D deficient patients and the disease outcome (DANDRIEUX 2016). Mildly elevated liver enzyme activities are often encountered on the biochemistry profile of dogs with CIE, and this might be due to a secondary impairment of the liver by the intestinal inflammation (reactive hepatitis). Lastly, hypomagnesemia and hypocalcemia have been reported in dogs with CIE, especially in Yorkshire terriers with PLE (KIMMEL et al. 2000).

2.1.1.4 Endoscopic Criteria

Intestinal biopsy is crucial to provide evidence of intestinal inflammation – a cornerstone of the diagnosis of CIE (DAY et al. 2008, WASHABAU et al. 2010). Biopsies can be obtained by flexible endoscopy, laparoscopy, or exploratory laparotomy (WASHABAU et al. 2010, ERDMANN and HEILMANN 2017). Endoscopy is the least invasive method of biopsy and

allows for direct visual assessment of mucosal changes and targeted sampling of mucosal tissue specimens (WASHABAU et al. 2010, JERGENS et al. 2016). Furthermore, endoscopy permits collection of multiple tissue specimens and thus increases the likelihood of detecting a localized disease (WASHABAU et al. 2010, JERGENS et al. 2016). However, one major limitation of endoscopic biopsies is the access to only certain segments of the gastrointestinal tract, which are the esophagus, stomach, duodenum, ileum, and colon. Also, endoscopic biopsies typically contain mucosa but do not allow for evaluation of lesions in the tunica muscularis. Thus, full-thickness biopsies are necessary if deeper layers of the mucosa or the entire wall of the intestine need to be evaluated (WASHABAU et al. 2010, JERGENS et al. 2016). The intestinal mucosa is evaluated endoscopically by assessing the mucosal appearance, friability, granularity, erosions or ulcerations, hyperemia, lymphatic dilatation, and possible mass lesions (SLOVAK et al. 2015, JERGENS et al. 2016). If no lesions are visible, the mucosa is considered as endoscopically normal. Friability describes immoderate bleeding induced by manipulation with the endoscope or the biopsy forceps (SLOVAK et al. 2015, JERGENS et al. 2016), whereas granularity refers to an altered texture of the mucosa (SLOVAK et al. 2015, JERGENS et al. 2016). Erosions are superficial mucosal defects with hemorrhage (SLOVAK et al. 2015, JERGENS et al. 2016), and hyperemia describes the degree of mucosal redness. Multifocal or diffuse white dots or speckles within the mucosa are defined as lymphatic dilatation or lymphangiectasia, and a mass is an abnormal growth of tissue extending into the gastrointestinal lumen (SLOVAK et al. 2015, JERGENS et al. 2016). In addition to the mucosal appearance, the ability to insufflate the bowel endoscopically is assessed (ALLENSPACH et al. 2007). SLOVAK et al. (2015) developed an endoscopic activity score for canine IBD including quantitative and qualitative assessment of friability, granularity, erosions and lymphatic dilatations. Such a score can aid in assessing the severity of endoscopic findings. Severe endoscopic disease in the duodenum has been reported to be significantly associated with a negative outcome (ALLENSPACH et al. 2007). It should be emphasized that there is no correlation between the severity of endoscopic and histologic lesions (ALLENSPACH et al. 2007, HEILMANN et al. 2014).

2.1.1.5 Histopathologic Criteria

In healthy dogs, the duodenal mucosa exhibits long, slender and uniform villi, and uniform crypts aligned perpendicularly to the surface (DAY et al. 2008). Lymphocytes and plasma cells occupy approximately 25% of the area of one 40x magnified microscopic field of the lamina propria of villi and there may be 1-2 lymphocytes detected between the crypts (DAY et al. 2008). Approximately 2-3 eosinophils per 40x magnified microscopic field are considered as a normal finding and neutrophils, which can be difficult to detect during routine

histopathology, should not be present in moderate or high numbers in the lamina propria under normal conditions (GERMAN et al. 1999, DAY et al. 2008). Figure 1 illustrates the histopathologic appearance of normal duodenal mucosa.

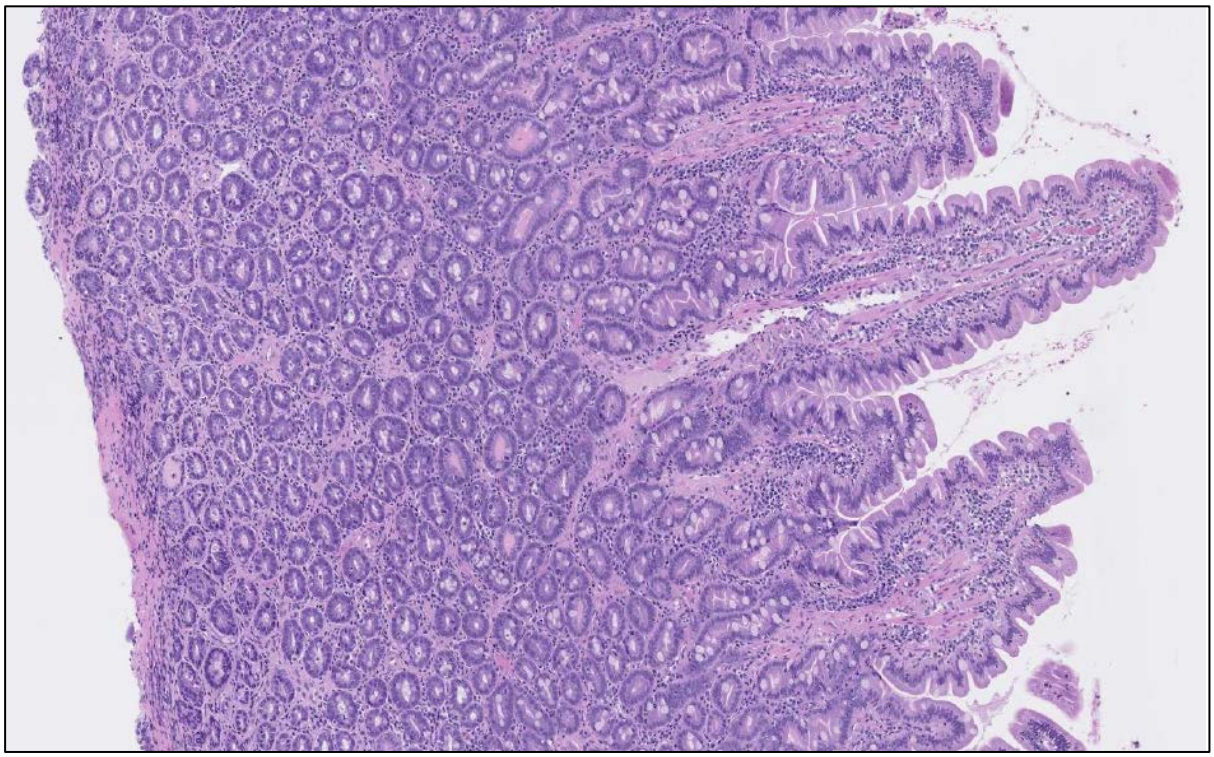

Figure 1. Duodenum with normal mucosa. This picture is printed by courtesy of Dr. Romy Heilmann; © 2014; Gastrointestinal Laboratory, Texas A&M University, College Station, TX, USA.

The colonic mucosa does not contain villi and is composed of a single layer of columnar epithelium, perpendicular and mainly uniform crypts, and numerous goblet cells (DAY et al. 2008). At most five lymphocytes and plasma cells should be present between crypts in healthy dogs. One or two eosinophils within the lamina propria are considered normal, and neutrophils should not be present in moderate or high numbers (GERMAN et al. 1999, DAY et al. 2008).

In addition to cellular infiltrates within the epithelium and the lamina propria, morphological abnormalities are assessed and graded to assess chronicity or further categorize inflammatory enteropathies (DAY et al. 2008, WASHABAU et al. 2010). Typical morphological changes of the duodenum include epithelial injury, villous stunting, crypt distention, lacteal dilation, and mucosal fibrosis (figures 2 - 4) (WASHABAU et al. 2010). In the colonic mucosa the presence of surface epithelial injury, crypt hyperplasia, crypt dilation and distortion, and mucosal fibrosis and atrophy are structural criteria that can aid in further classifying the disease (figures 5 and 6) (WASHABAU et al. 2010). Based on the severity of inflammatory cell infiltration and structural abnormalities, a distinction is made between mild,

moderate and severe / marked histologic disease in accordance with published guidelines (DAY et al. 2008, WASHABAU et al. 2010). Interestingly, histologic lesion severity has been demonstrated to not be correlated with clinical response to treatment and overall patient outcome (CRAVEN et al. 2004, ALLENSPACH et al. 2007, GARCIA-SANCHO et al. 2007, SCHREINER et al. 2008). Biochemical variables, namely hypoalbuminemia and hypocobalaminemia, and the severity of endoscopic lesions in the duodenum appear to be more relevant instead and have been shown to be associated with a negative outcome (ALLENSPACH et al. 2007).

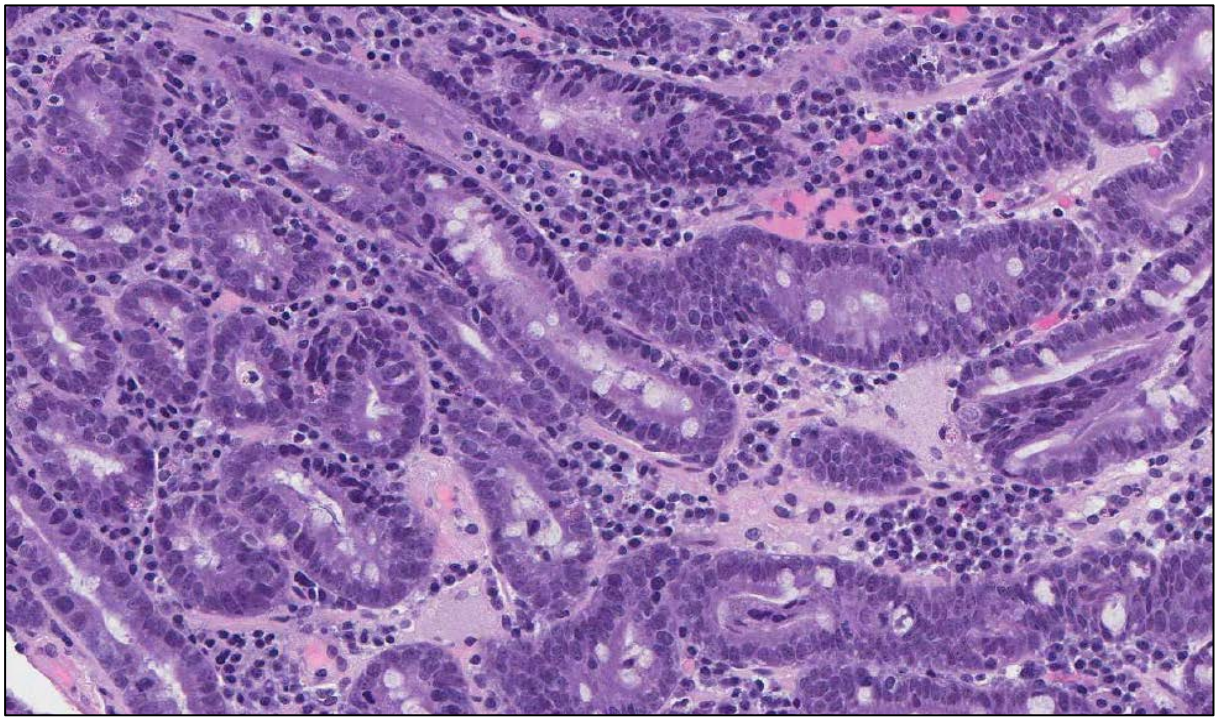

Figure 2. Duodenum with moderate crypt hyperplasia and a moderate increase in plasma cells in the lamina propria. Courtesy of Dr. Romy Heilmann; © 2014; Gastrointestinal Laboratory, Texas A&M University, College Station, TX, USA.

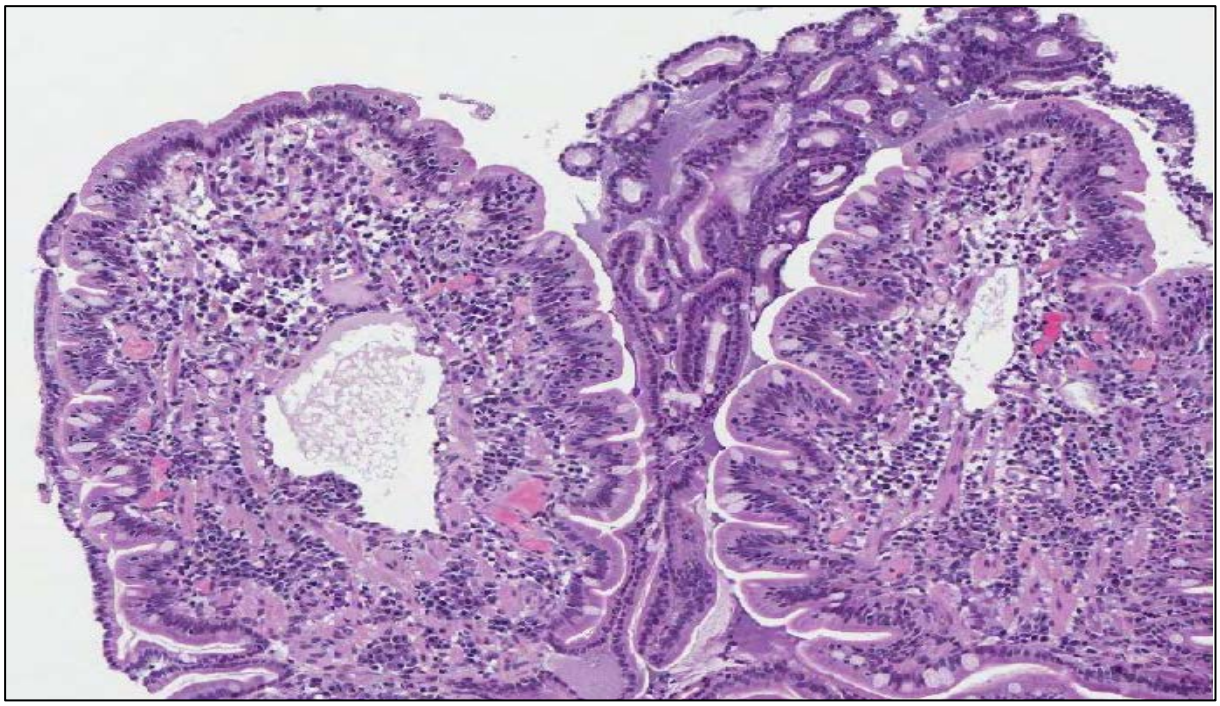

Figure 3. Image of duodenal villi. The villi are slightly widened and have a dilated lacteal. There are infiltrates of lymphocytes and plasma cells. © 2014 Dr. Romy Heilmann; Gastrointestinal Laboratory, Texas A&M University, College Station, TX, USA.

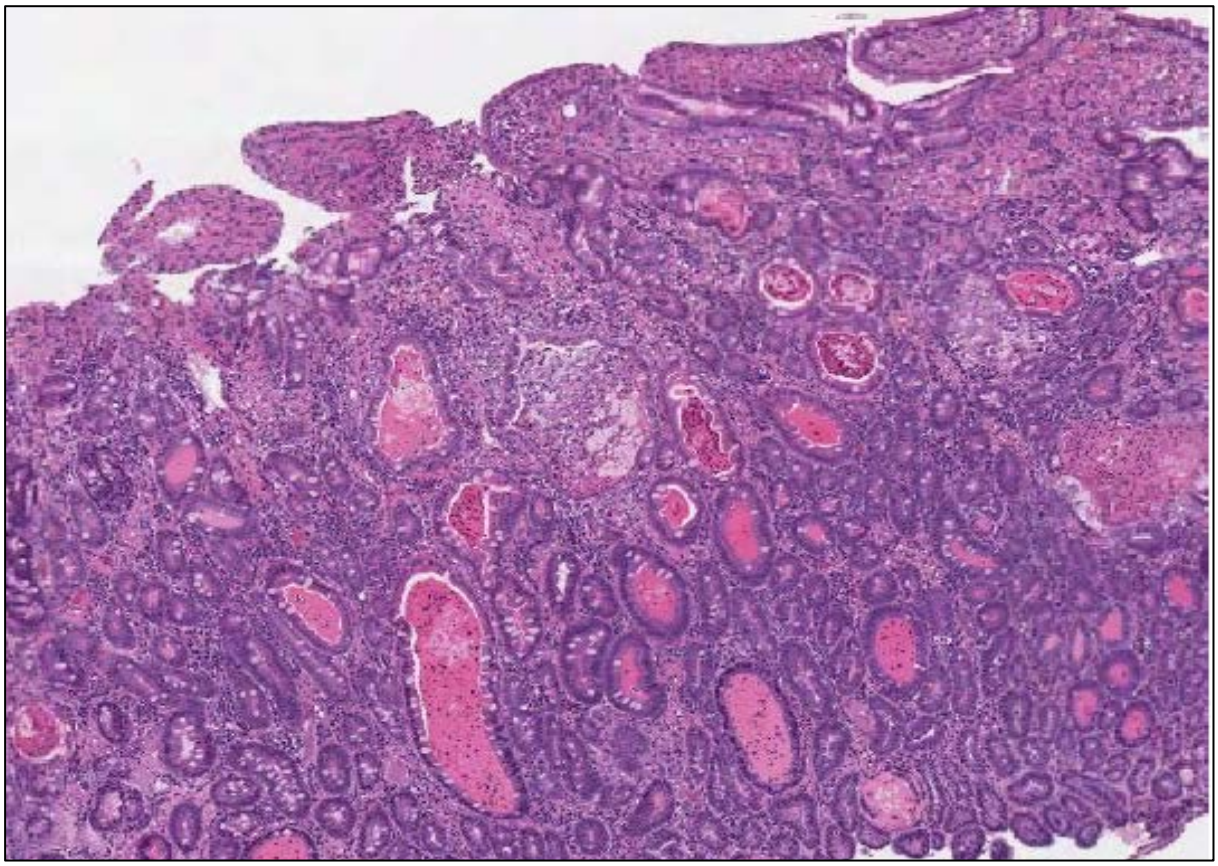

Figure 4. Diffuse, severe, chronic mixed (lymphoplasmacytic, eosinophilic and neutrophilic) duodenitis with numerous crypt abscesses and severe villous atrophy. This picture is printed by courtesy of Dr. Romy Heilmann; © 2014; Gastrointestinal Laboratory, Texas A&M University, College Station, TX, USA.

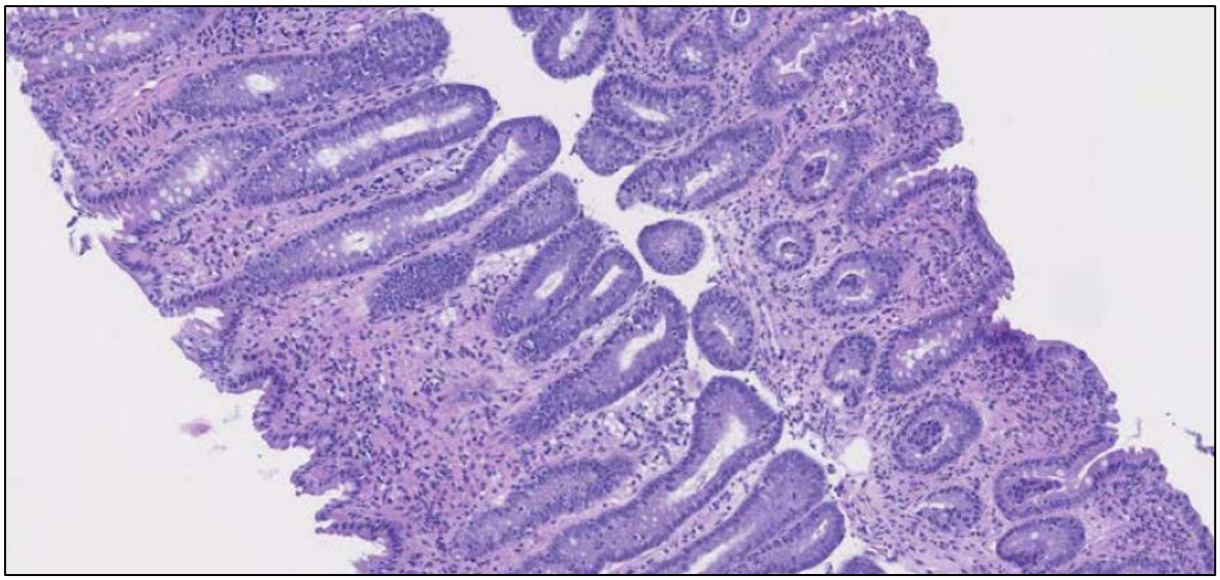

Figure 5. Image of the colon with loss of cryptal structure with some mild hyperplasia and distortion, decreased goblet cells and mild to moderate inflammatory infiltration of the lamina propria. © 2014 Dr. Romy Heilmann; Gastrointestinal Laboratory, Texas A&M University, College Station, TX, USA.

It should be emphasized that the different chronic inflammatory enteropathies – food-responsive diarrhea (FRD), antibiotic-responsive diarrhea (ARD), and idiopathic inflammatory bowel disease (IBD) – can display very similar histologic lesions, and consequently, these conditions cannot be distinguished by histopathology alone (GERMAN et al. 2003a, ALLENSPACH et al. 2007, DANDRIEUX 2016).

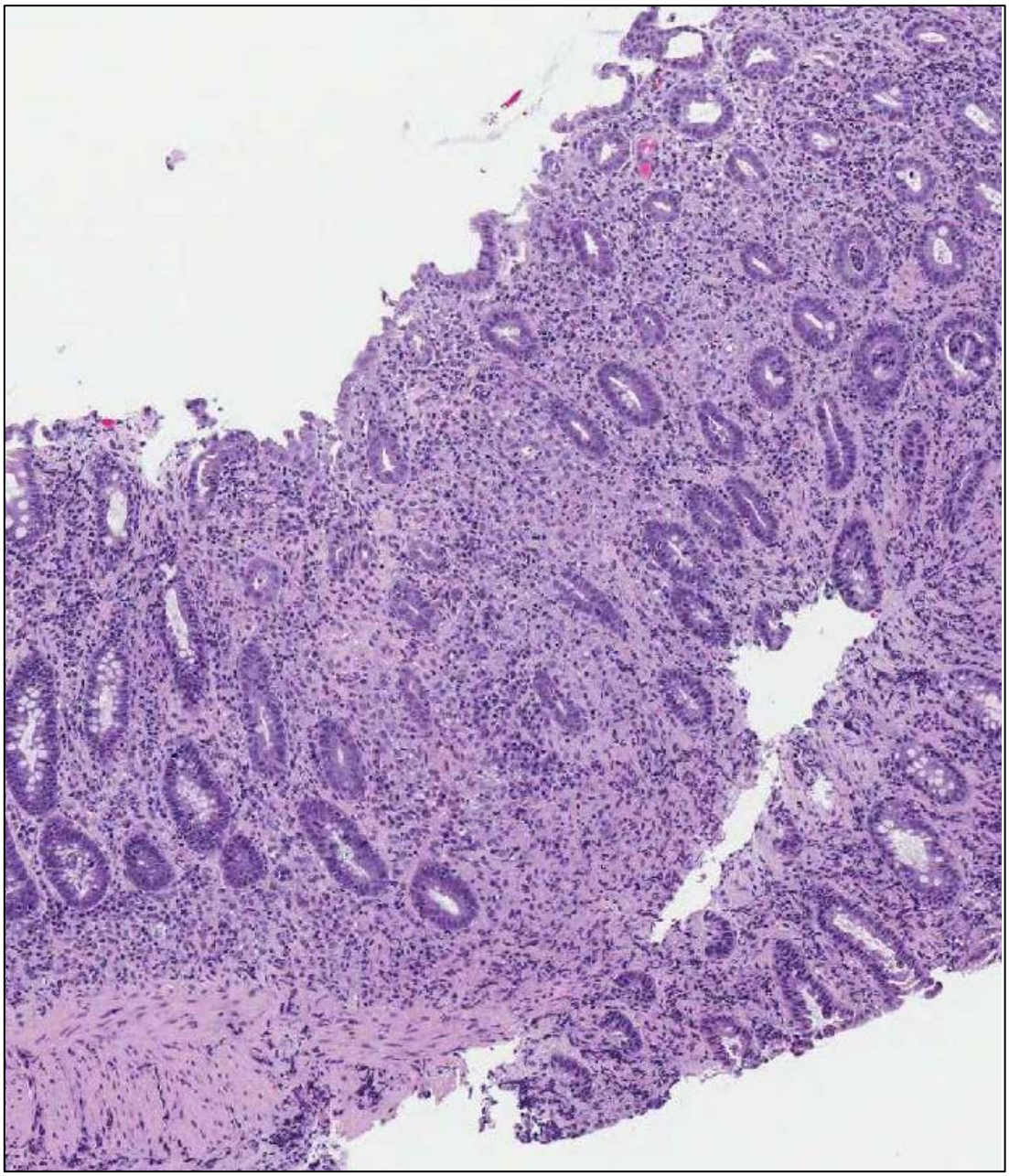

Figure 6. Extensive infiltration and expansion of the colonic lamina propria by numerous histiocytes with multifocal surface erosion and occasional ulceration. Courtesy of Dr. Romy Heilmann; © 2014; Gastrointestinal Laboratory, Texas A&M University, College Station, TX, USA.

2.1.2 Idiopathic Inflammatory Bowel Disease

2.1.2.1 Pathogenesis

Idiopathic inflammatory bowel disease (IBD) is a collective term to describe gastrointestinal disorders that have the following characteristics (GERMAN et al. 2003a, WASHABAU et al. 2010, DANDRIEUX 2016):

- chronic gastrointestinal signs (duration of > 3 weeks)
- histologic evidence of mucosal inflammation
- thorough diagnostic investigation and exclusion of other possible causes for chronic gastrointestinal signs
- failure to respond to appropriately designed therapeutic trials (i.e. antiparasitic medication, elimination diet, antimicrobial treatment)
- response to anti-inflammatory and / or immunosuppressive drugs.

The specific triggers leading to the development of IBD are still unknown, but recent studies in both human and companion animal medicine support the hypothesis of IBD pathogenesis involving the interplay of genetic host susceptibility, the intestinal microenvironment, dietary and environmental factors, and an exaggerated immune-response (SIMPSON and JERGENS 2011, DANDRIEUX 2016).

The fact that certain breeds of dogs are predisposed to develop IBD supports the hypothesis of a genetic effect on the development of IBD. Such predispositions have been reported, for instance, for the German shepherd dog (KATHRANI et al. 2010, KATHRANI et al. 2011), the Basenji (BREITSCHWERDT et al. 1980, MACLACHLAN et al. 1988), the Norwegian Lundehund (KOLBJØRNSEN et al. 1994), the Chinese Shar-Pei (GRÜTZNER et al. 2010) and the Yorkshire terrier (KIMMEL et al. 2000, SIMMERSON et al. 2014). However, until now no breed-independent etiologic genetic mutations or defects have been detected (SIMPSON and JERGENS 2011).

Toll-like receptors (TLR) are pattern-recognition receptors that recognize microbe-associated molecular pattern molecules (MAMPs) as well as endogenous damage-associated molecular patterns (DAMPs) (HEILMANN and ALLENSPACH 2017). These receptors are part of the innate immune system. The upregulation of selected TLRs (TLR2, TLR4, and TLR9) and the downregulation of TLR5 in duodenal and colonic mucosa in dogs with IBD as well as the absence of significant changes after therapy despite obvious clinical improvement of dogs might suggest a genetic predisposition (BURGENER et al. 2008, MCMAHON et al. 2010, ALLENSPACH et al. 2010b). *NOD2* (nucleotide oligomerization domain 2) is another innate

immune receptor that has been investigated for a possible association with the pathogenesis of canine IBD following the discovery that mutations in the *NOD2* gene are associated with Crohn's disease in humans (XAVIER and PODOLSKY 2007, PACKEY and SARTOR 2008). Single nucleotide polymorphisms have also been detected in the canine *NOD2* gene, and their increased frequency in dogs with IBD would also suggest a genetic basis of host susceptibility to the development of IBD (KATHRANI et al. 2014).

The intestinal microbiome might also play a role in the development of IBD either indirectly via interaction with dysregulated pattern recognition receptors or directly via pathogenic mechanisms. Invasive *E.coli* for example have been demonstrated to be linked to the development of histiocytic ulcerative colitis in Boxer dogs. This condition represents a special form of CIE that often responds to treatment with fluoroquinolones (MANSFIELD et al. 2009). Further, current consensus is that a change in the intestinal microbial composition also plays a role in the pathogenesis of IBD. Intestinal dysbiosis characterized by an increase in Proteobacteria (SUCHODOLSKI et al. 2012a, SUCHODOLSKI et al. 2012b, HONNEFFER et al. 2014, MINAMOTO et al. 2015) and a decrease in *Faecalibacterium* spp. *(SUCHODOLSKI et al. 2012b)* and Clostridia (SUCHODOLSKI et al. 2010) has been revealed in dogs with IBD.

Dietary constituents may also contribute to gastrointestinal inflammation. One example is the enteropathy induced by ingestion of gluten diagnosed in the Irish Setter breed (GARDEN et al. 2000). Mucosal reactions seen during gastrointestinal endoscopy have been reported in Soft Coated Wheaten Terriers after intragastric administration of food extracts containing milk, and adverse food reactions were detected in these dogs during a dietary trial with food ingredients such as corn, tofu, cottage cheese, milk, or lamb (VADEN et al. 2000). Furthermore, a study by MANDIGERS et al. (2010) revealed that dogs with chronic enteropathies showed a positive response to a hydrolyzed protein diet. All three studies support the hypothesis that dietary components present an important factor in the pathogenesis of IBD.

All these potential causes of IBD, either individually or as part of a complex interaction among these factors, can result in an exaggerated immune-response with resultant chronic inflammation. However, it is reasonable to assume that an overreactive immune system in itself could be the source of inflammation. Until today, the exact causal relationships between potential factors of IBD pathogenesis remain elusive (SIMPSON and JERGENS 2011) and further research is needed to evaluate these aspects.

2.1.2.2 Diagnosis and Treatment

A diagnosis of IBD can only be reached when several criteria have been considered. First, the patient signalment and a thorough history to evaluate clinical signs, diet, eating behavior, and environmental factors are gathered. Second, a thorough physical examination is performed, and the possibility of endoparasite infections and other causes of chronic gastroenterocolitis need to be excluded via clinicopathologic tests, diagnostic imaging, and fecal examination (SIMPSON and JERGENS 2011, DANDRIEUX 2016). Third, empirical therapeutic treatment with antiparasitic and antimicrobial medication and at least three to four weeks of a dietary trial with an elimination diet have been performed and did not lead to a significant clinical improvement (WASHABAU et al. 2010, SIMPSON and JERGENS 2011, DANDRIEUX 2016). Fourth, gastrointestinal biopsy specimens have been obtained and revealed histologic evidence of inflammation in accordance with published guidelines (DAY et al. 2008, WASHABAU et al. 2010), whereby lymphoplasmacytic inflammation represents the most common histologic finding. Lastly, resolution or significant improvement of clinical signs (clinical remission) is seen after anti-inflammatory and / or immunosuppressive treatment (WASHABAU et al. 2010, SIMPSON and JERGENS 2011, DANDRIEUX 2016). Additionally, several biomarkers (e.g., cobalamin, methylmalonic acid, folate, alpha$_1$-proteinase inhibitor, calprotectin, S100A12, 3-bromotyrosine, and N-methylhistamine) have been evaluated in dogs with CIE in the past decade. Despite not being pathognomonic, these biomarkers can aid in the diagnostic evaluation and patient monitoring in dogs with CIE (HEILMANN and STEINER 2018).

Immunosuppressant drugs represent the mainstay of treatment in dogs with idiopathic IBD (DANDRIEUX 2016) and are given in addition to a dietary modification. Thus, idiopathic IBD is also referred to as immunosuppressant-responsive enteropathy (IRE). Glucocorticoids are currently the first-line option in the treatment of primary inflammatory and autoimmune diseases in dogs (VIVIANO 2013), and prednisolone at a dose of 1 mg/kg given twice daily (BID) has been proven to be an effective induction treatment in dogs with CIE (JERGENS et al. 2010). Commonly, this dose is slowly tapered over several weeks to the lowest effective dose (top-down approach) once the patient's clinical signs are controlled (JERGENS et al. 1992). Nevertheless, some dogs develop severe adverse effects (e.g., iatrogenic hyperadrenocorticism with corresponding clinical signs, gastrointestinal ulceration, pronounced muscular atrophy, opportunistic infections) (VIVIANO 2013) or are refractory to glucocorticosteroids and thus require other or additional immunosuppressant drugs to control clinical signs of idiopathic IBD (DANDRIEUX 2016). Cyclosporine, a calcineurin inhibitor, has been shown to be an effective alternative in the treatment of CIE (ALLENSPACH et al. 2006, ALLENSPACH et al. 2007). Cyclosporine is widely used for its steroid-sparing effect in combination with prednisolone or as a rescue treatment option in steroid-refractory IBD

cases (ALLENSPACH et al. 2006, HALL and GERMAN 2010). The main disadvantage of using cyclosporine is its rather high expense when compared to glucocorticosteroids. A recent study, evaluating chlorambucil in combination with prednisolone in the treatment of dogs with CIE and protein-losing enteropathy, revealed chlorambucil to also be an effective immunosuppressive treatment option in dogs with IBD (DANDRIEUX et al. 2013). However, larger studies comparing the effect of different immunosuppressants (e.g., cyclosporine, mycophenolate, azathioprine, chlorambucil, or leflunomide) in a randomized and blinded fashion are currently lacking. Thus, the usefulness of these treatment options or even a combination thereof in the management of canine CIE cannot be definitively determined at this time. Because of a hypercoagulable state in dogs with PLE the treatment of those dogs with IBD and PLE should also include a platelet aggregation inhibitor (e.g., clopidogrel) (GOODWIN et al. 2011, ERDMANN and HEILMANN 2017).

Some cases of IBD may not respond to treatment regardless of the specific treatment plan and therefore carry a guarded prognosis (DANDRIEUX 2016). Risk factors that have been reported to be associated with a negative outcome are a high clinical disease activity index, severe endoscopic disease in the duodenum, hypoalbuminemia, and hypocobalaminemia (ALLENSPACH et al. 2007). The severity of clinical disease and response to treatment can be assessed using established clinical scoring systems – the canine IBD activity index (CIBDAI) (JERGENS et al. 2003) and the canine chronic enteropathy clinical activity index (CCECAI) (ALLENSPACH et al. 2007) (q.v. chapter 2.1.1.2 clinical disease activity).

2.1.3 Food-Responsive Diarrhea

2.1.3.1 Pathogenesis

Food-responsive diarrhea (FRD) is the most common cause of CIE. In a recent study, 64% of dogs with CIE were diagnosed with FRD (ALLENSPACH et al. 2016). Dogs with FRD are often younger than dogs with IBD, and they typically present with a lower clinical disease activity index than dogs with IBD (ALLENSPACH et al. 2007, ALLENSPACH et al. 2016, DANDRIEUX 2016). FRD is characterized by an adverse reaction to food that is a repeatable, undesired response to any dietary component (HALL and GERMAN 2010). This adverse reaction can be the result of an immunologic or a non-immunologic reaction. The former is considered as a true food hypersensitivity whereas the latter, non-immunologic reactions, are referred to as food intolerance and include food poisoning, pharmacologic intolerance (e.g., methylxanthines in chocolate poisoning), or gluttony (HALL and GERMAN 2010).

Canine dietary hypersensitivity can be an IgE-mediated immediate reaction (type I) to ingested food allergens. However, non-IgE-mediated reactions such as a type II (immune-complex mediated) or a delayed type IV reaction may also occur (DAY 2005, HALL and GERMAN 2010). Mechanisms that – either alone or in combination – have been hypothesized to be responsible for the breakdown of gastrointestinal immune tolerance include an altered presentation of dietary antigens to the mucosal immune system, a dysregulated immune system, and an inadequate mucosal barrier function (GERMAN et al. 2003a, HALL and GERMAN 2010). Regardless of the underlying pathomechanism, adverse food reactions result in nonspecific gastrointestinal signs such as vomiting, diarrhea, abdominal pain, inappetence, or weight loss (HALL and GERMAN 2010).

2.1.3.2 Diagnosis and Treatment

The diagnosis of FRD is established based on the response to dietary modification, particularly the exclusion of the offending dietary antigen (HALL and GERMAN 2010, DANDRIEUX 2016). However, in most instances the offending antigen remains unknown. Therefore, feeding an elimination diet consisting of a novel source of protein and carbohydrate is advised for an exclusion dietary trial (SIMPSON and JERGENS 2011, DANDRIEUX 2016). A proper elimination diet trial requires strict feeding of only the elimination diet. Commercially available hydrolyzed protein diets have been shown to be an effective alternative to the feeding of a novel protein elimination diet (MARKS et al. 2002, MANDIGERS et al. 2010). In order to confirm the diagnosis of FRD, a challenge with the original diet needs to be performed once clinical remission is achieved, and this dietary challenge should demonstrate a relapse of clinical signs. For obvious reasons, many owners and clinicians are reluctant to perform such a provocation diet trial. Hence, affected dogs will often be kept on the elimination diet or transitioned from a hydrolyzed diet to a novel protein diet without rechallenge (HALL and GERMAN 2010, SIMPSON and JERGENS 2011).

In addition to these diagnostic aspects, dietary modification is also the cornerstone of FRD treatment in dogs (SIMPSON and JERGENS 2011, DANDRIEUX 2016). An excellent long-term response has been reported both with novel antigen diets (ALLENSPACH et al. 2007) and hydrolyzed protein diets (MANDIGERS et al. 2010, ALLENSPACH et al. 2016). Commercially available hydrolyzed protein diets are nutritionally well-balanced and can be used as a maintenance diet. If the owner prefers long-term feeding of a home-cooked meal with a novel antigen, a veterinary nutritionist should be consulted to formulate a recipe and ensure a balanced diet. If this option is considered, both home-cooked and commercial hydrolyzed diets are equivalent treatment options. Eventually some dogs with FRD can return to their original diet without relapse of clinical signs. However, this is a trial-and-error

approach (DANDRIEUX 2016) and the owners should be educated about the different treatment options.

2.1.4 Antibiotic-Responsive Diarrhea

Antibiotic-responsive diarrhea (ARD) currently represents the third subgroup of canine CIE. Several hypotheses exist on the pathogenesis of ARD, such as its development secondary to defects in the mucosal barrier function, abnormal mucosal immune responses, or qualitative changes in the intestinal bacterial microbiome, but the exact mechanisms are still to be resolved (HALL and GERMAN 2010, HONNEFFER et al. 2014). Adherent-invasive *E. coli* (AIEC) have been documented within the intestinal lamina propria of Boxer dogs (MANSFIELD et al. 2009), French Bulldogs (MANCHESTER et al. 2013), and mastiffs (STOKES et al. 2001) affected with histiocytic ulcerative colitis (formerly known as granulomatous colitis or Boxer colitis). Because resolution of clinical signs and eradication of mucosal AIEC was achieved after extensive treatment of 6 to 10 weeks (MANCHESTER et al. 2013, DANDRIEUX 2016) with enrofloxacin, histiocytic ulcerative colitis is currently the only CIE for which a causative agent has been found (MANSFIELD et al. 2009, MANCHESTER et al. 2013).

Generally, dogs diagnosed with ARD are younger than dogs with IBD (GERMAN et al. 2003a, ALLENSPACH et al. 2016), and a breed predisposition has been identified in the German shepherd dog (BATT et al. 1991, ALLENSPACH et al. 2010b). As part of the sequential diagnostic investigation of dogs with CIE, an antimicrobial trial is often instituted after appropriately designed dietary trials have failed. Metronidazole and tylosin are the most commonly used antibiotics (WESTERMARCK et al. 2005, DANDRIEUX 2016). Several studies have shown that tylosin can be effective for the treatment of chronic diarrhea, referred to as tylosin-responsive diarrhea (TRD) by some authors (WESTERMARCK et al. 2005, KILPINEN et al. 2011, KILPINEN et al. 2014, KILPINEN et al. 2015). However, relapses of clinical signs occur frequently after cessation of antibiotic treatment (WESTERMARCK et al. 2005, ALLENSPACH et al. 2016). The exact mode of action of antibiotics in the absence of obvious pathogenic bacteria remains unclear. Immunomodulatory and anti-inflammatory properties in addition to a modification of the intestinal microbiota (e.g., a shift towards more beneficial bacterial species) are suspected to be associated with the positive effects of antibiotics (CAO et al. 2006, SUCHODOLSKI et al. 2009, KILPINEN et al. 2015, DANDRIEUX 2016). However, it needs to be emphasized that the response to antimicrobial treatment is often rather short-lived, and with the emerging concerns regarding antimicrobial resistance, the true usefulness of antibiotic trials in the

treatment of CIE has come under scrutiny. Thus, other treatment options should always be considered first, unless a causative agent has been detected or is strongly suspected.

2.2 The Canine Intestinal Microbiome

2.2.1 Methods to Evaluate the Intestinal Microbiome

The intestinal microbiome is composed of all microorganisms harbored in the gastrointestinal (GI) tract – which includes bacteria, viruses, fungi, and protozoa (SUCHODOLSKI 2016). Because bacteria represent the most abundant microorganisms in the intestinal lumen (SWANSON et al. 2011), the attention of this study is turned to the intestinal bacteria only. The term “microbiota” is generally accepted to describe bacterial communities, and therefore the author adopted this term for the subsequent analyses.

It should be emphasized that, to date, there is no single detection method that is considered a “gold standard” for the evaluation of the intestinal microbiota (SUCHODOLSKI 2016). In the past, culture-dependent techniques were widely used to assess microbial communities (HANDL et al. 2011, SUCHODOLSKI 2016). However, it is now known that at most 20% of the intestinal bacteria can be cultivated (SUCHODOLSKI 2016). This low detection rate results mainly from the fact that the canine intestinal tract is composed of mostly anaerobic bacteria, which are very vulnerable and elusive to standard laboratory conditions. Furthermore, growth requirements of many bacteria are unknown (SUCHODOLSKI 2016). Yet, bacterial cultures are vitally important for the detection of specific enteropathogens, such as *Salmonella* spp., and antimicrobial sensitivity testing (SUCHODOLSKI 2016).

The discovery of new molecular techniques has promoted further research on the intestinal microbiota and their functional implications. Identification of previously undetected bacteria is now possible. Fluorescence in situ hybridization (FISH) uses fluorescent dye-labelled oligonucleotide probes that are hybridized to bacterial ribosomal RNA sequences and can be subjected to subsequent microscopic evaluation (LANGER-SAFER et al. 1982, SUCHODOLSKI 2016). FISH analysis allows identification of most bacterial species and is the currently most accurate method for quantification of bacteria. Additionally, FISH can help to determine whether bacteria are within the intestinal lumen, adherent to cells, or invading the mucosa (SUCHODOLSKI 2016). When using quantitative real-time PCR (qPCR), bacteria are detected in real-time mode by fluorescent dye-labeled primers (SUCHODOLSKI 2016). Both FISH and qPCR require probes and assay kits that are specifically designed for the bacterial group of interest. Next generation sequencing technologies (e.g., 454-pyrosequencing, Illumina) are high-throughput sequencing platforms that permit massive parallel analyses and provide a large quantity of data (LIU et al. 2012, GOODWIN et al. 2016). Here, universal primers are used to amplify bacteria and these PCR amplicons are

then analyzed with a high-throughput sequencer (SUCHODOLSKI 2016). Both 454-pyrosequencing and Illumina implement the technology of sequencing by synthesis and detect bases as they are embedded into the growing DNA or RNA strands (LIU et al. 2012, GOODWIN et al. 2016). Pyrosequencing technology detects pyrophosphates which are released during nucleotide incorporation (LIU et al. 2012). When using Illumina, a charge-coupled device detects the individually cleaved fluorescent dyes of the four deoxynucleotides after these have been incorporated into an extending complementary nucleotide strand (LIU et al. 2012, GOODWIN et al. 2016). Currently, next generation sequencing of 16S ribosomal RNA (16S rRNA) genes plays an important role in the further investigation of the intestinal microbiota. It allows for a general impression of the quantities and proportions of all bacterial groups present within the intestine (SUCHODOLSKI 2016). However, bacterial groups presenting at a very low abundance may be missed when using high-throughput sequencing. Hence, the additional use of group-specific PCR primers is advised (SUCHODOLSKI 2016).

The term metagenomics refers to the shotgun sequencing of the whole metagenome. This approach evaluates DNA from the entire bacterial community by analyzing short reads of all existing genomes without prior PCR amplification (SEGATA et al. 2013, GUARD and SUCHODOLSKI 2016). Gathered sequences can then be annotated using a reference catalog of bacterial genes and genomes. This approach yields both identification of bacterial species and information about the function of microbial communities (GUARD and SUCHODOLSKI 2016), and as a consequence is currently one of the most promising methods. However, this method is very cost-intensive and requires advanced bioinformatics (SUCHODOLSKI 2016). Hence, metagenomics is currently only used in advanced sciences.

In summary, the best approach to unravelling the intestinal microbiota would be a coordinated approach using a combination of all currently available molecular tools.

2.2.2 The Intestinal Microbiome in Healthy Dogs

The intestinal microbiota is of major relevance for host health. It aids in proper epithelial development and the defense against enteropathogens, for example by maintaining an intact epithelial barrier or by competitive exclusion of pathogens (NEISH 2009, SUCHODOLSKI 2016). It also provides stimuli for the intestinal immune system and can modulate the immune response (NEISH 2009, SUCHODOLSKI 2016). Furthermore, the intestinal microbiota provides nutritional factors to the host, including the synthesis of essential vitamins and degradation of complex polysaccharides to short-chain fatty acids, which serve as an energy source for the host (NEISH 2009, SWANSON et al. 2011). Short-chain fatty acids also regulate intestinal motility (SUCHODOLSKI 2016) and have anti-inflammatory effects by inducing immunoregulatory T cells (ARPAIA et al. 2013).

In addition, primary bile acids that enter the lumen of the colon are converted to secondary bile acids by intestinal bacteria (PAVLIDIS et al. 2015, SUCHODOLSKI 2016). Conversion of bile acids is another example of a beneficial relationship between the intestinal microbiota and the host. Secondary bile acids are anti-inflammatory and can modulate insulin secretion and glucose metabolism, and are therefore essential contributors to gut homeostasis (PAVLIDIS et al. 2015, SUCHODOLSKI 2016).

According to studies using the methodology of bacterial culture, the intestinal tract of healthy dogs harbors a large bacterial load approximating 10^2 - 10^5 and up to 10^9 colony forming units (CFU)/g intestinal juice in the small intestine (GERMAN et al. 2003b, SUCHODOLSKI 2016). Due to a physiological increase in microbial numbers and diversity along the GI tract (SUCHODOLSKI et al. 2005), numbers of luminal bacteria are even higher in the colon, ranging from 10^8 to 10^{11} CFU/g (MENTULA et al. 2005).

In healthy dogs, Firmicutes, Bacteroidetes, Proteobacteria and Fusobacteria are the most abundant bacterial phyla (SUCHODOLSKI et al. 2008, XENOULIS et al. 2008, SUCHODOLSKI et al. 2009, HANDL et al. 2011) in the intestine. While Firmicutes are found in all parts of the intestine, Fusobacteriales and Bacteroidales were only sporadically demonstrated in the proximal intestine, but were the most abundant bacterial orders in the intestinal content of the ileum and colon (MENTULA et al. 2005, SUCHODOLSKI et al. 2008). Two studies evaluating fecal bacterial communities reported Bacteroidetes to be the second most abundant phylum detected in feces of healthy dogs (MIDDELBOS et al. 2010, HANDL et al. 2011). Proteobacteria, which also include *E. coli*-like organisms, on the other hand, are abundant especially in the small intestine and present at lower abundances in the colon (SUCHODOLSKI et al. 2008). One study reported Proteobacteria to be the second most abundant phylum in the duodenum (XENOULIS et al. 2008) and another study reported Proteobacteria to be the most abundant phylum in the jejunum (SUCHODOLSKI et al. 2009). Of all detected phyla, Firmicutes is overall the most abundant phylum (SUCHODOLSKI et al. 2008, XENOULIS et al. 2008, SUCHODOLSKI et al. 2009, HANDL et al. 2011) and within this phylum the order Clostridiales has been shown to be the most diverse and prevalent in the canine intestinal tract (SUCHODOLSKI et al. 2008, HANDL et al. 2011). Members of the order Clostridiales form several clusters, of which Clostridium cluster XI and Clostridium cluster XIVa represent the predominant clusters (SUCHODOLSKI et al. 2008, HANDL et al. 2011). Genera associated with Clostridium cluster XI were more abundant in the small intestine, whereas genera affiliated with Clostridium cluster XIVa (e.g., *Eubacterium* spp., *Ruminococcus* spp.) dominated in the colon (SUCHODOLSKI et al. 2008). Both clusters have been shown to produce short-chain fatty acids (SUCHODOLSKI 2016) and therefore might have a beneficial impact on the host (NEISH 2009, ARPAIA et al. 2013,

SUCHODOLSKI 2016). Besides Clostridiales, another prevalent bacterial order in the canine intestine is Lactobacillales. Members of this order (e.g., Lactobacillaceae, Enterococcaceae, and Streptococcaceae) are present in large abundances in the duodenum, jejunum (SUCHODOLSKI et al. 2008, XENOULIS et al. 2008), and colon (SUCHODOLSKI et al. 2008), whereas lower abundances are found in the ileum (SUCHODOLSKI et al. 2008). In addition to the aforementioned phyla, Spirochaetes and Actinobacteria (especially Coriobacteriales) also present at significant abundances (XENOULIS et al. 2008, SUCHODOLSKI et al. 2009). One study also identified four phyla in the jejunum of dogs that had not been identified in the canine intestine before: Tenericutes, Cyanobacteria, Verrucomicrobia and Chloroflex. All of these four phyla were only present in very low abundances (SUCHODOLSKI et al. 2009). *Bifidobacterium* spp. belong to the phylum Actinobacteria and are considered beneficial microorganisms, which are also used as probiotics (e.g., the strain *Bifidobacterium sp. animalis*) (SCHMITZ and SUCHODOLSKI 2016). Interestingly, the detection of this genus has not been consistently reported. While one study reported an abundance of up to 10^{10} CFU/ml in the colon (DAVIS et al. 1977), *Bifidobacterium* spp. was not observed at all in two more recent studies evaluating intestinal content (DELLES et al. 1994, SUCHODOLSKI et al. 2008) and in one study evaluating the duodenal mucosa (DELLES et al. 1994). *Bifidobacterium* spp. were observed only at low abundance in one study evaluating canine feces (HANDL et al. 2011). Thus, future studies using advanced molecular tools and specific primers are warranted to reveal the true prevalence of this genus.

2.2.3 The Intestinal Microbiome in Dogs with CIE

Similar to the findings in human medicine (RIOUX et al. 2005, XAVIER and PODOLSKY 2007, DUBOC et al. 2013), evidence has also grown in veterinary medicine that, in addition to other factors, host susceptibility (ALLENSPACH et al. 2010b, KATHRANI et al. 2012, KATHRANI et al. 2014) and the intestinal microbiota (XENOULIS et al. 2008, ALLENSPACH et al. 2010b, SUCHODOLSKI et al. 2012a, SUCHODOLSKI et al. 2012b, HONNEFFER et al. 2014, MINAMOTO et al. 2015) play a pivotal role in the pathogenesis of inflammatory bowel disease (IBD). Alterations in the interaction between the host's enteric microbiota and the intestinal mucosa (e.g., abnormal barrier function, altered innate and adaptive immune responses) (XAVIER and PODOLSKY 2007) and the functional profile of the microbiota appear to contribute to the development of IBD (DUBOC et al. 2013, SUCHODOLSKI 2016). To date, several studies have evaluated the intestinal microbial composition in dogs with IBD and generally have revealed an intestinal dysbiosis (SUCHODOLSKI 2016).

Proteobacteria, for example members of the Enterobacteriaceae (XENOULIS et al. 2008), *Brevundimonas* spp., *Brucella* spp., *Pseudomonas* spp. (SUCHODOLSKI et al. 2010), *Acinetobacter* spp. and *Diaphorobacter* spp. (SUCHODOLSKI et al. 2012a), were found to be enriched in duodenal brush cytologies (XENOULIS et al. 2008) and mucosal tissue biopsies (SUCHODOLSKI et al. 2010, SUCHODOLSKI et al. 2012a) in dogs with IBD, whereas members of Fusobacteria, Bacteroidetes, Spirochaetes and Clostridiaceae were significantly more abundant in the duodenum of healthy dogs (XENOULIS et al. 2008, SUCHODOLSKI et al. 2010). The study by XENOULIS et al. (2008) also reported significantly lower species richness in the duodenum of dogs with IBD. Another study examining microbial alterations in the ileal and colonic mucosa in dogs with chronic enteropathies revealed an increased total number of bacteria, but a decrease in *Bacteroides* spp. within the colonic mucosa in dogs with IBD compared to healthy dogs (CASSMANN et al. 2016). The number of total bacteria attached to the colonic mucosa was also positively correlated with clinical disease severity, assessed by the CIBDAI score, in dogs with IBD. Furthermore, dogs with IBD had increased numbers of Enterobacteriaceae and *E. coli* compared to dogs with other chronic enteropathies, such as histiocytic ulcerative colitis or malignant alimentary tract neoplasia (CASSMANN et al. 2016). Evaluation of the fecal microbiota did not reveal significant differences in the number of observed species between dogs with IBD and healthy control dogs (SUCHODOLSKI et al. 2012b, MINAMOTO et al. 2015, XU et al. 2016), but differentially expressed bacterial taxa could be identified. Again, Proteobacteria, particularly Gammaproteobacteria, were reported to be more abundant in dogs with IBD, whereas Erysipelotrichia (e.g., *Turicibacter* spp.), Clostridia (e.g., *Blautia* spp., *Faecalibacterium* spp.) and Bacteroidia were found at low abundance in dogs with IBD when evaluated using 454-pyrosequencing (MINAMOTO et al. 2015). On the contrary, another study using FISH analysis found *Bacteroides* spp. in larger numbers in dogs with chronic diarrhea than in healthy dogs (JIA et al. 2010). This discrepancy might be explained by the use of different methods to detect and quantify bacteria. SUCHODOLSKI et al. (2012b) showed differences in the presence of *Faecalibacterium* spp. and Fusobacteria between dogs with clinically active and clinically insignificant IBD, assessed by the CIBDAI score. Both taxa were decreased in times of clinically active disease (SUCHODOLSKI et al. 2012b). Similarly, the fecal quantity of *Lactobacillus* spp. was negatively correlated with CCECAI scores in another study (XU et al. 2016).

Currently, it is unclear if these microbial changes seen in dogs with IBD are more of a cause or the result of the disease. However, it is assumed that changes in the microbial composition also implicate changes of the microbial functional profile (e.g., altered amino acid metabolism, reduced production of short-chain fatty acids, altered bile acid metabolism)

(GUARD et al. 2015, MINAMOTO et al. 2015, SUCHODOLSKI 2016), which may possibly contribute to both the development and sustainment or perpetuation of IBD.

Lastly, a fecal dysbiosis index (DI) has been published recently. Based on the assessment of the fecal abundance of eight bacterial groups shown to be frequently altered in dogs with CIE (total bacteria, *Faecalibacterium* spp., *Turicibacter* spp., *Escherichia coli*, *Streptococcus* spp., *Blautia* spp., *Fusobacterium* spp. and *Clostridium hiranonis*) by quantitative PCR and their analysis using a complex mathematical algorithm, this index distinguishes normobiosis (DI < 0) from dysbiosis (DI > 0) and is ultimately able to separate healthy dogs from dogs with CIE (74% sensitivity, 95% specificity, area under the curve (AUC) of 0.93) (ALSHAWAQFEH et al. 2017). The DI may be a valuable tool to monitor treatment response and resultant changes of the fecal microbiota, allowing for an individual approach to patients.

2.3 The Canine Lipidome

2.3.1 Methods to Evaluate the Lipidome

Lipids are defined as hydrophobic or amphipathic organic molecules that commonly dissolve in organic solvents (FAHY et al. 2005). According to the LIPID MAPS “Comprehensive Classification System for Lipids”, these molecules are classified into eight categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides (FAHY et al. 2005, FAHY et al. 2009). This enormous structural diversity of lipids, which is further increased by a large multitude of building blocks that can be either incorporated into or separated from the core structure of these molecules, poses a challenge for the research of lipids and their metabolism. However, recent advances in technology render lipidomics, the quantitative description and analysis of lipids and their functions possible (VAN MEER 2005, LAM and SHUI 2013, LI et al. 2014). In general, lipidomics can be classified as a targeted and untargeted approach. The targeted approach evaluates specific lipids, whereas the non-targeted approach aims at investigating the entire lipid structure of a given sample (LAM and SHUI 2013, ASTARITA and OLLERO 2015). Regardless of the approach, lipidomics usually combine several analytical steps, including the extraction, separation, and analysis of lipids (LI et al. 2014). Lipids are most commonly separated via chromatographic techniques, such as gas chromatography (GS) or liquid chromatography (LC) (LAM and SHUI 2013, LI et al. 2014). Mass-spectrometry (MS) is the major constituent of lipid analysis but a complete characterization of lipids usually requires a combination of MS, LC, and ionization techniques (WENK 2010, LI et al. 2014, ASTARITA and OLLERO 2015). In addition, the large structural and chemical diversity of lipids makes it almost impossible to analyze the entire lipidome of a specific sample using a single analytical method (WENK 2010, LI et al. 2014). Thus, a series of several different techniques and

analyses are usually necessary. Conventional ionization techniques are electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI), atmospheric pressure chemical ionization, and electron impact ionization (LAM and SHUI 2013, LI et al. 2014). Electrospray ionization is considered a soft-ionization technique, which is superior to electron impact ionization because it does not require derivatization of fatty acids. Thus, ESI offers an advantage over other techniques for analysis of non-volatile thermally unstable molecules (HO et al. 2003, LI et al. 2014). Currently, ESI is an important ionization source for mass spectrometry and coupling this technique with high performance liquid chromatography allows for the analysis of a larger number of lipid classes (HO et al. 2003, LI et al. 2014). Tandem mass spectrometry describes a series of two quadrupole mass spectrometers that are separated by a collision cell (VOGESER and PARHOFER 2007). A combination of liquid chromatography and tandem mass spectrometry is often used for the analysis of lipids with a low abundance (LI et al. 2014). The availability of all these analytical methods is an important prerequisite for the feasibility of lipidomics, even to the extent of a “shotgun” approach to lipidomics. However, despite the overall progress in the analysis of lipids, the field of lipidomics is still in its infancy and further advances in analytical methods, for example methods to discriminate between isobaric or isometric lipids (LI et al. 2014), are still needed to fully understand the features and complexity of lipid molecules.

2.3.2 Functions of Lipids and their Importance to Health

Similar to proteins, lipids are crucial for many physiologic processes to be maintained in cells, tissues, and organs. It has been known for decades that lipids are the basic components of bio- and cellular membranes and that lipids serve as an important means for energy storage (figure 7) (WENK 2005, VAN DER MEER-JANSSEN et al. 2010). Recently, additional important biological functions of lipids have been being discovered. Because second messengers can be derived from membrane lipids (BERRIDGE 1993), lipids play a central role in a number of signaling pathways. Furthermore, lipids have been shown to regulate membrane trafficking, for example by the recruitment of proteins or by modulation of the membrane structure and function (HAUCKE and DI PAOLO 2007). Due to these versatile functions it is obvious that lipid homeostasis is pivotal to the maintenance of health.

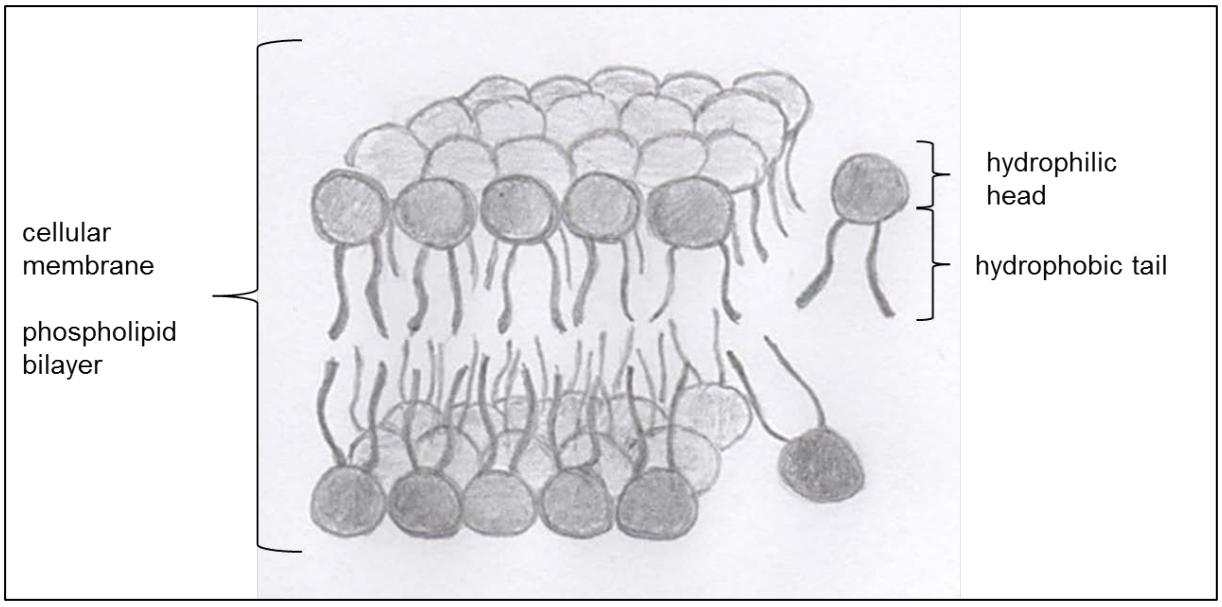

Figure 7. Schematic diagram of a cellular membrane comprising phospholipids.

In human medicine, impairment of lipid homeostasis has been shown to be involved in the pathogenesis of several diseases, such as infectious diseases, metabolic syndrome, neurodegenerative diseases, and cancer (FIORENZA et al. 2000, WENK 2005, VAN DER MEER-JANSSEN et al. 2010, LAM and SHUI 2013). Several pathogens are capable of exploiting the intricacy of lipids and their functions resulting in the modulation of host cell responses and survival of the pathogen. Such tactics of the pathogens include the molecular mimicry of lipids (both by *de novo* synthesis and incorporation of the host's lipids), alterations of fatty acid compositions of membranes (e.g., *Plasmodium* spp. in erythrocytes, HIV-1 in lymphocytes), and injury to cellular or vacuolar host membranes through the action of phospholipases (VAN DER MEER-JANSSEN et al. 2010).

The metabolic syndrome refers to a combination of dyslipidemia, systemic hypertension, and hyperglycemia or a disturbance in glucose homeostasis (BEILBY 2004). Until today, it is not fully understood how alterations in the lipidome result in metabolic syndrome, but recent studies have begun to shed some light on this association (LAM and SHUI 2013). Increased concentrations of circulating triglycerides and cholesterol, for example, can modulate cellular lipid metabolism and result in intracellular lipid accumulation and impairment of organ function (LAM and SHUI 2013). In line with that, it has been shown that the reduction of sphingolipid synthesis has a positive effect on tissue insulin sensitivity (VAN EIJK et al. 2009). Thus, medical intervention to alter levels of circulating lipids might be an important aspect in the treatment of metabolic disorders (LAM and SHUI 2013).

Alterations in cerebral lipid metabolism have been recognized to contribute, for example, to the pathogenesis of Alzheimer's disease. Cholesterol, for instance, increases the secretion of the amyloid-ß peptide – an important factor in the development of Alzheimer's disease. Thus,

increased concentrations of cholesterol and amyloid-ß peptide might promote the development of Alzheimer's disease. Similarly, ceramide, which also stimulates the production of amyloid-ß peptide, has been reported to be increased in the cortex and the cerebrospinal fluid of patients with Alzheimer's disease (CUTLER et al. 2004, LIU et al. 2007, HE et al. 2010, LIU and ZHANG 2014).

Differences in the lipoprotein profile have also been reported between patients with cancer and patients with non-neoplastic diseases (FIORENZA et al. 2000), and an inverse association has been shown to exist between total cholesterol concentrations and the risk of breast cancer (TOUVIER et al. 2015). However, the true role of lipids in the pathogenesis and the risk of cancer is still unknown and further research is warranted to further understand the role of lipids in cancerogenesis.

In addition to the aforementioned diseases, dyslipidemia has also been demonstrated in patients with IBD (AGOURIDIS et al. 2011). One study reported total and low-density lipoprotein (LDL)-cholesterol to be decreased in patients with active Crohn's disease and ulcerative colitis, and total and high-density lipoprotein (HDL)-cholesterol was inversely correlated with markers of inflammation, such as C-reactive protein, in patients with Crohn's disease (ROMANATO et al. 2009). Another study revealed that patients with Crohn's disease have lower serum concentrations of total cholesterol than patients with ulcerative colitis (FAN et al. 2015). That study further found that several lipids, especially alkylphospholipids and plasmalogens, were significantly associated with Crohn's disease, whereas no lipid class and only few lipid species varied significantly between patients with ulcerative colitis and healthy control subjects. That study also suggested that decreased concentrations of plasmalogens may contribute to the development of human IBD (FAN et al. 2015). Generally, a decrease in circulating total cholesterol and either no difference or an increase in triglycerides accounted for part of the dyslipidemia present in patients with IBD (LEVY et al. 2000, RIPOLLES PIQUER et al. 2006, SAPPATI BIYYANI et al. 2010). However, whether dyslipidemia is a cause or rather a consequence of IBD needs to be investigated in future studies.

Similar to human medicine, lipid homeostasis and alterations thereof have also attracted great attention in veterinary medicine. To date, several studies have described the basic processes of the canine lipid metabolism (JOHNSON 2005, XENOULIS and STEINER 2010, 2015). Triglycerides and cholesterol are transported in the plasma via lipoproteins. Among these lipoproteins are chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) (JOHNSON 2005, XENOULIS and STEINER 2010, 2015). Chylomicrons are part of the exogenous pathway of lipid metabolism, and are generated in the intestinal epithelial cells and carry dietary lipids to muscles and fatty tissue. VLDL, LDL, and HDL, on the other hand, are part of the endogenous metabolic

pathway. VLDL and HDL are formed in the liver and consecutively altered in the plasma. For instance, triglycerides are separated from VLDLs by lipoprotein lipase and hepatic triglyceride lipase, which transforms VLDLs into VLDL remnants and LDLs. While LDL then transports cholesterol to the tissue, HDL is considered the “good lipoprotein” as it assembles tissue cholesterol and returns it to the liver (JOHNSON 2005, XENOULIS and STEINER 2010, 2015).

Hyperlipidemia, which is defined as an increased serum concentration of lipids (triglycerides and / or cholesterol) (XENOULIS and STEINER 2010, 2015), is a relevant disorder in dogs (JOHNSON 2005, XENOULIS and STEINER 2010, 2015). It can be either a primary or a secondary condition. Hypertriglyceridemia of the Miniature Schnauzer is the most studied form of primary hyperlipidemia in dogs (XENOULIS et al. 2007), but primary hyperlipidemia has also been documented in other breeds, such as hypercholesterolemia of the Briard, hyperlipidemia of the Shetland Sheepdog, hypercholesterolemia in Dobermann and Rottweilers (WATSON et al. 1993, MORI et al. 2010, XENOULIS and STEINER 2015). Overall, secondary hyperlipidemia is far more common than primary hyperlipidemia and has been reported with several diseases. Endocrine disorders, such as hypothyroidism, hyperadrenocorticism, and diabetes mellitus, are the most common underlying diseases associated with hyperlipidemia (XENOULIS and STEINER 2010, 2015, SEAGE et al. 2018). Further, obesity, protein-losing nephropathy, cholestatic liver disease, high-fat diets, and certain drugs (e.g., glucocorticoids, phenobarbital) have been linked to the development of hyperlipidemia (LITTMAN et al. 2000, JEUSETTE et al. 2005, JOHNSON 2005, KLUGER et al. 2008, XENOULIS and STEINER 2015). Hyperlipidemia is also believed to be associated with pancreatitis in dogs (HESS et al. 1998), but recent studies suggest that severe hyperlipidemia in dogs diagnosed with pancreatitis presents rather a separate condition or consequence of an additional endocrine disorder (XENOULIS and STEINER 2015). Regardless of its cause, hyperlipidemia itself may result in pancreatitis, gallbladder mucocele, vacuolar hepatopathy, ocular disease, and neurological signs including seizures (VITALE and OLBY 2007, ZARFOSS and DUBIELZIG 2007, XENOULIS and STEINER 2010, XENOULIS et al. 2010, XENOULIS and STEINER 2015). One recent study also reported low-grade systemic inflammation to be present in some Miniature Schnauzers with idiopathic hyperlipidemia (HEILMANN et al. 2019). Thus, severe hyperlipidemia requires therapeutic intervention, which should include treatment of the underlying disease, use of an (ultra)low-fat diet, supplementation of omega-3 fatty acids and, if further medical management is necessary, also lipid-lowering medications such as fibrates or nicacin (XENOULIS and STEINER 2015).

Alterations in lipid profiles have also been described in several other canine diseases. YILMAZ and SENTURK (2007) reported lower serum levels of total cholesterol, HDL- and LDL-cholesterol and higher serum levels of triglycerides in dogs diagnosed with parvoviral enteritis than in a control group of dogs. Also, serum HDL concentrations were found to be lower in non-survivors when compared to survivors and control dogs. However, further research is warranted to fully understand the role and prognostic use of hypocholesterolemia in dogs with parvovirus infection and evidence of sepsis (YILMAZ and SENTURK 2007). Dyslipidemia has also been reported in dogs with chronic kidney disease and nephrotic syndrome. Especially an increase in LDL and VLDL concentrations and a decrease in HDL concentrations accounted for the altered lipoprotein profile (BEHLING-KELLY 2014). Another study revealed differences in lipid species between tissue samples of canine transitional cell carcinomas of the urinary bladder and samples of adjacent normal tissue, with members of sphingolipids and glycerophospholipids being present at significantly different concentrations (DILL et al. 2009). Considering all of these findings, lipid homeostasis and dyslipidemia appears to play an important role in the pathogenesis of many canine diseases. To date, research on lipids in canine medicine is still in its early stages, thus opening new avenues for future studies investigating the functions and the potential diagnostic and therapeutic implications of the canine lipidome. The author postulates that the canine circulating lipid profile differs between different subclassifications of canine CIE and also within each disease category with regard to the treatment status.

3 OWN PUBLICATIONS

3.1 Comparison of the Intestinal Mucosal Microbiota in Dogs Diagnosed with Idiopathic Inflammatory Bowel Disease and Dogs with Food-Responsive Diarrhea before and after Treatment

My own contribution to this first study of the dissertation consisted of the administration of this project including the coordination of different responsibilities and interpretation of the results considering clinical aspects and the relevance of our findings for the scientific and also the general veterinary community. I compiled all the data analyzed by Illumina sequencing with the help of my co-authors in this study, related those to findings of previous studies, and wrote this manuscript.

This is a pre-copyedited, author-produced version of an article accepted for publication in FEMS Microbiology Ecology following peer review. The version of record Katja Kalenyak et al. Comparison of the intestinal mucosal microbiota in dogs diagnosed with idiopathic inflammatory bowel disease and dogs with food-responsive diarrhea before and after treatment. FEMS Microbiology Ecology (2018) 94 (2): fix173 is available online at: https://doi.org/10.1093/femsec/fix173; doi: 10.1093/femsec/fix173.

Comparison of the intestinal mucosal microbiota in dogs diagnosed with idiopathic inflammatory bowel disease and dogs with food-responsive diarrhea before and after treatment

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One sentence summary: This is the first study to evaluate the intestinal mucosal microbiota of dogs with idiopathic inflammatory bowel disease and dogs with food-responsive diarrhea before and after treatment

ABSTRACT

We report the first study to evaluate the intestinal mucosal microbiota of dogs with inflammatory bowel disease (IBD) and dogs with food-responsive diarrhea (FRD) before and after treatment. It was hypothesized that differences in the microbial composition exist between both disease groups and within groups pre- vs. post-treatment. Duodenal and colonic biopsies were obtained endoscopically from 24 dogs (15 FRD, 9 IBD) before and after treatment. The intestinal microbiota was evaluated by Illumina sequencing of the bacterial 16S rRNA gene. The global bacterial composition did not differ between IBD and FRD dogs, nor between treatment status. However, several bacterial taxa showed a difference in abundance. Comparing disease groups, an unclassified genus of Neisseriaceae was abundant in the duodenum in the IBD group, whereas *Bilophila* occurred more frequently in the duodenum and *Burkholderia* in the colon of FRD dogs. Comparing the microbiota pre- and post-treatment revealed *Enterococcus*, *Corynebacterium* and Proteobacteria to be enriched in the duodenum of FRD dogs pre-treatment, while *Bacteroides* was abundant in the colon post-treatment. In dogs with IBD, *Bacteroides* also reached significant abundance in the colon post-treatment. In conclusion, some differences in individual bacterial taxa were identified between IBD and FRD dogs and between treatment status.

Keywords: mucosal microbiota; chronic enteropathies; canine; treatment; duodenum; colon

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INTRODUCTION

Chronic enteropathies are a group of common disorders in dogs and are characterized by persistent or recurrent clinical signs of gastrointestinal disease, including diarrhea, vomiting, weight loss, inappetence or borborygm and flatulence (German, Hall and Day 2003; Hall and German 2010; Dandrieux 2016). Based on the response to treatment, chronic enteropathies are classified as food-responsive diarrhea (FRD), antibiotic-responsive diarrhea (ARD) or idiopathic inflammatory bowel disease (IBD) (Hall and German 2010; Dandrieux 2016). In dogs with FRD, clinical signs resolve after dietary modification to a novel source of protein and carbohydrate or to a hydrolyzed protein diet (Hall and German 2010; Mandigers et al. 2010). Dogs with ARD respond to dietary management and antibiotic treatment, for example, with tylosin (German, Hall and Day 2003; Westermarck et al. 2005; Kilpinen et al. 2011). Idiopathic IBD is defined by the aforementioned chronic gastrointestinal signs and confirmation of intestinal inflammation by histology (German, Hall and Day 2003; Hall and German 2010; Dandrieux 2016). Until today, the pathogenesis of chronic enteropathies, and in particular idiopathic IBD, is not fully understood. The upregulation of Toll-like receptors in dogs with idiopathic IBD and the lack of significant changes after treatment in the face of obvious clinical improvement (Burgener et al. 2008) suggest a genetic susceptibility as a contributing factor. This concept is further supported by the differential expression of Toll-like receptors 4 and 5 in German shepherd dogs with chronic enteropathies (Allenspach et al. 2010). Additionally, the identification of non-synonymous single nucleotide polymorphisms in exon 3 of the *NOD2* gene (Kathrani et al. 2014) is in line with the findings of studies on the pathogenesis of IBD in human patients where several susceptibility genes could be identified (e.g., *NOD2* gene) in patients with Crohn's disease (Xavier and Podolsky 2007). Furthermore, dietary and environmental factors are suspected to be main contributors in the development of idiopathic IBD (Hall and German 2010; Dandrieux 2016). While the exact mechanisms of host–microbe interactions remain elusive, evidence has grown to support that the intestinal microbiota plays a major role in the pathogenesis of idiopathic IBD (Xavier and Podolsky 2007; Suchodolski et al. 2010, 2012a, 2012b; Minamoto et al. 2015; Cassmann et al. 2016; Vázquez-Baeza et al. 2016). Moreover, advanced scientific techniques, such as next-generation sequencing, metagenomics and metabolomics, can facilitate research on the clinical relevance of the intestinal microbiota and their metabolites. Several studies have assessed the gastrointestinal microbiome in healthy dogs, dogs with idiopathic IBD and dogs with acute diarrhea. An interindividual diversity in the abundance of bacterial classes has been shown to exist even in healthy dogs (Handl et al. 2011; Garcia-Mazcorro et al. 2012; Guard and Suchodolski 2016), making inferences on the significance of changes in the intestinal microbiota somewhat difficult. Regardless of this overall variability in the microbial abundances, several studies have revealed an intestinal dysbiosis in dogs

with idiopathic IBD. The intestinal dysbiosis was reflected mainly by an increase in Proteobacteria and a decrease in *Faecalibacterium* when compared to healthy dogs (Suchodolski et al. 2010, 2012a, 2012b; Minamoto et al. 2015; Vázquez-Baeza et al. 2016).

However, these recent studies have evaluated the fecal or duodenal mucosal microbiota of dogs with idiopathic IBD at a single timepoint only. To date, there is no study reported evaluating the mucosal microbiota in dogs with chronic enteropathies both before and after treatment. Furthermore, only little information is available on the differences in the intestinal microbiota of dogs diagnosed with IBD or FRD. Thus, the aims of this study were (i) to compare the duodenal and colonic mucosal microbiota between dogs with IBD and dogs with FRD, and (ii) to evaluate the effect of successful treatment on the microbial composition by comparing the mucosal microbiota of each dog before and after treatment. Our hypotheses were that (i) the mucosal microbial composition differs between the two disease classifications, and (ii) the mucosal microbiome also differs within each disease group depending upon the treatment status.

MATERIALS AND METHODS

Animals and study protocol

Duodenal and colonic mucosal biopsies were retrieved from a former study on canine chronic enteropathies. The exact study protocol has been published elsewhere (Burgener et al. 2008; Dumusc et al. 2014) and is briefly summarized here. Dogs with chronic gastrointestinal signs were prospectively enrolled between 2006 and 2008. All of the dogs had diarrhea with or without vomiting for at least 6 weeks. Further inclusion criteria were the absence of an identifiable underlying disorder; histopathological evidence of intestinal inflammation; and no treatment with antibiotics, corticosteroids and/or antacids 2 weeks prior to enrollment into the study. Most dogs had already received dietary modifications prior to referral. Potential underlying disorders were ruled out by a CBC, biochemistry profile, measurement of serum trypsin-like-immunoreactivity (TLI), cobalamin and folate, ACTH stimulation test, urinalysis, parasitic and bacterial fecal examination, abdominal ultrasound and endoscopy of the gastrointestinal tract. Since the specific canine pancreatic lipase was not easily available between 2006 and 2008, the diagnosis of pancreatitis was ruled out considering amylase, lipase, TLI and sonographic findings. Also, all dogs received treatment with fenbendazole (50 mg/kg daily for 5 days) regardless of the fecal examination. All owners signed a letter of consent, and the study was reviewed and approved by the Cantonal Committee of Animal Experimentation, Bern, Switzerland.

A clinical disease severity score (canine IBD activity index [CIBDAI]) (Jergens et al. 2003) was assigned to each dog before and after treatment. The more detailed canine chronic

enteropathy clinical activity index (CCECAI) (Allenspach et al. 2007) was published during the course of this study and was assigned in some dogs. However, to obtain consistent results for all dogs, the CCECAI score was not further evaluated in the current study. In addition, every dog was categorized to have either mainly upper or lower gastrointestinal signs, or a combination of both. Both a gastroduodenoscopy and colonoscopy were performed in each dog enrolled in the study.

Following the complete diagnostic evaluation, including gastrointestinal endoscopy, all dogs received a standardized elimination diet for 14 days. The elimination diet was a selected protein diet based on codfish and rice only, with codfish being a novel source of protein for all dogs enrolled in the study. That dry diet was specially produced for the current study (Biomill SA, Granges-Marnand, Switzerland). The diet was tested for contamination, and the adequacy of the nutritional composition was calculated by a veterinary nutritionist. Owners were thoroughly instructed on the principle of an elimination diet, including the importance of strictly feeding the prescribed diet. If clinical signs improved significantly or resolved within the first 14 days of feeding the diet, dogs were assigned to the FRD group. Although it is possible that few dogs with FRD had not yet responded, this length of the elimination trial was chosen according to previous publications that showed that most dogs with FRD usually respond within the first 2 weeks of a dietary trial (Marks, Laflamme and McAloose 2002; Allenspach et al. 2007; Gaschen and Merchant 2011; Allenspach, Culverwell and Chan 2016). Dogs that did not respond to the elimination diet alone were assigned to the idiopathic IBD/steroid-responsive group and received additional prednisolone (1 mg/kg BID) for 14 days followed by a slow tapering of the dose. Cyclosporine (5 mg/kg SID) or other immunosuppressants (e.g., budesonide) were given to dogs that did not improve on prednisolone.

Post-treatment assessment included the re-evaluation of the CIBDAI score and a repeat gastrointestinal endoscopy in all dogs. The FRD group of dogs was reassessed 4 weeks after starting the elimination diet, whereas the IBD group of dogs was re-evaluated at 10 weeks after starting treatment with prednisolone.

Gastrointestinal endoscopy and histopathological evaluation

Details on the endoscopic and the histopathological evaluation have been published elsewhere (Burgener et al. 2008). Briefly, mucosal biopsy specimens were retrieved from the duodenum (~10 cm below the caudal duodenal flexure) and the colon (the middle portion of the descending colon) or from areas with visible lesions. Samples were placed in 4% neutral-buffered formalin for 48 h before being embedded in paraffin and subsequently prepared for histopathological evaluation. In addition, three endoscopic biopsy samples were obtained

from each intestinal section and were placed in RNA-later solution followed by storage at -70°C until DNA extraction.

The endoscopic biopsies were examined histologically by a board-certified pathologist blinded to the number of endoscopy, diagnosis and treatment. The pathologist assigned a histologic lesion score reflecting the degree of inflammation and cellular infiltration (Jergens et al. 1992). Updated histopathological guidelines were published by the World Small Animal Veterinary Association Gastrointestinal Standardization Group in 2008 (Day et al. 2008). Similar to the CCECAI, these guidelines were not used in this study in order to apply the same histopathological standards to all dogs.

Bacterial 16S rRNA gene quantitation and sequencing

Genomic DNA was extracted from duodenal and colonic biopsies using a commercially available DNA extraction kit (PowerSoil®, Mo Bio, Carlsbad, CA, USA) according to the manufacturer's instructions. Amplification and sequencing of the V4 variable region (primers 515F/806R) of the 16S rRNA gene was performed on a MiSeq (Illumina) at the Molecular Research MR DNA laboratory (www.mrdnalab.com, Shallowater, TX, USA) as described previously (Bell et al. 2014). The software Quantitative Insights Into Microbial Ecology (QIIME) v.1.8 (http://www.qiime.org) was used for processing and analysis of sequences (Caporaso et al. 2010). The raw sequence data were de-multiplexed, and low-quality reads were filtered using default parameters. Chimeric sequences were detected using USEARCH (Edgar 2010) and were removed prior to further analysis. The remaining sequences were then assigned to operational taxonomic units (OTUs) using an open-reference OTU picking protocol in QIIME against the Greengenes (DeSantis et al. 2006) database (v.13.8). Prior to the downstream steps, sequences that were assigned as chloroplast, mitochondria and low abundance OTUs were removed. The rarefaction depth was set at 15 170 sequences per sample for colon samples and 2530 sequences per sample for duodenal samples. The sequences were deposited in the Sequence Read Archive under the following accession number: SRP103535.

Within-sample diversity was estimated with the alpha diversity indices Chao1, Shannon and Observed OTUs. Beta diversity, which refers to the similarity between samples and potential clustering patterns between sample groups, was visualized using principal coordinate analysis plot based on weighted and unweighted UniFrac distances.

Statistical analysis

Statistical analyses were performed using JMP® Pro v.12. A Shapiro-Wilk test was used to assess the data distribution for normality. Because the majority of the datasets did not meet

the assumption of a normal distribution, comparisons of the alpha diversity and the bacterial taxa between FRD dogs and IBD dogs were performed using a Mann-Whitney U test. A Wilcoxon signed-rank test was used for comparison of paired samples (pre- and post-treatment) within each disease group. A Benjamini-Hochberg false discovery rate was used to control for multiple testing. P- and q-values < 0.05 were considered statistically significant. The analysis of similarities (ANOSIM) function in the statistical software package PRIMER 6 (PRIMER-E Ltd, Luton, UK) was used on the weighted and unweighted UniFrac distance matrix to determine if any groups of samples contained significantly different bacterial communities. Linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al. 2011) was performed to identify bacterial groups that were significantly associated with disease classification and/or treatment status. LEfSe was used in the Galaxy workflow framework with the parameters set at $\alpha = 0.01$, LDA score = 2.0.

RESULTS

Dogs

Twenty-four dogs were included in the study: fifteen of these dogs responded to the dietary modification only (FRD group) and nine dogs needed additional immunosuppressant treatment (IBD group). Basic characteristics of all study dogs included in the study are summarized in Table 1. Two IBD dogs were panhypoproteinemic and were classified as having protein-losing enteropathy (PLE) as a result of severe lymphoplasmacytic inflammation due to idiopathic IBD. Both dogs responded to immunosuppressant therapy. One of these dogs, however, developed severe side effects while treated with prednisolone and was switched to budesonide (dosage 3 mg/m² SID), which was well tolerated.

In one dog diagnosed with FRD, duodenal biopsy samples were not sufficient for Illumina sequencing. Because duodenum and colon were analyzed separately, the colonic biopsies of this dog were still included in the analysis. In another dog with a diagnosis of FRD, the post-treatment sample did not meet the rarefaction depth that had been set for the analysis. Hence, this dog was excluded from the within-group evaluation of the effect of treatment on the mucosal microbial composition.

Sequence analysis

The sequence analysis yielded a total of 5 436 076 quality sequences for all analyzed samples (n = 96, mean ± SD = 55 877 ± 39 144). The average of Good's coverage of all samples was 97.3 ± 0.4% (mean ± SD., ranging from 96.3% to 98.2%).

Table 1. Characteristics of dogs (n = 24) enrolled in the study.

Disease	Breed	Age	Sex	Weight (kg)	BCS	CIBDAI
IBD	Shar Pei	4 y	f	12.4	2/9	9
IBD	Golden Retriever	6 y 10 mo	mn	36.5	6/9	7
IBD, PLE	Beauceron	4 y	fs	28.9	na	14
IBD, PLE	Bernese Mountain Dog	5 y	fs	35.5	na	12
IBD	Am. Cocker Spaniel	3 y 7 mo	mn	10.8	5/9	6
IBD	Mixed breed medium size	12 y 10 mo	mn	27.4	5/9	3
IBD	Cavalier King Charles Spaniel	4 y 6 mo	m	8.6	5/9	4
IBD	Malinois	2 y 8 mo	mn	32.6	4/9	15
IBD	Mixed breed medium size	2 y 11 mo	fs	20.3	5/9	4
FRD	Mixed breed medium size	3 y	mn	30.0	7/9	9
FRD	Yorkshire Terrier	8 y 6 mo	fs	2.9	6/9	6
FRD	French Bulldog	1 y 4 mo	m	14.6	5/9	8
FRD	Weimaraner	2 y	fs	23.0	4/9	4
FRD	Tervuren/Irish Wolfshound	9 mo	f	25.5	4/9	5
FRD	Samoyed/Border Collie/Swiss Mountain Dog	5 y 10 mo	mn	23.0	4/9	7
FRD	Cairn Terrier	3 y	m	9.4	na	4
FRD	Golden Retriever	1 y 2 mo	f	20.1	3/9	11
FRD	West Highland White Terrier	1 y	f	6.4	6/9	4
FRD	Labrador	2 y	m	46.0	6/9	9
FRD	Berger Blanc Suisse	2 y	fs	32.0	na	8
FRD	Pomeranian	10 mo	f	1.8	5/9	2
FRD	Labrador	11 y 2 mo	mn	32.5	6/9	4
FRD	Mixed breed large size	6 y 2 mo	fs	49.0	7/9	1
FRD	Newfoundland	6 y 9 mo	m	44.2	4/9	4

The canine inflammatory bowel disease activity index (CIBDAI) refers to the clinical activity score at the first visit.
The body condition score (BCS) refers to the body condition score of the first visit.
y, year; mo, months; f, female; m, male; n, neutered; s, spayed; na, not available.

Microbial communities in dogs with IBD or FRD

Diversity analysis

Alpha diversity, as described by species richness, Chao 1 and Shannon diversity index, was not significantly different between dogs with IBD and dogs with FRD in neither the duodenum nor colon (Fig. 1, Table 2). Also, within each disease group, significant differences were not seen before and after treatment (Fig. 2, Table 2).

Additionally, principal coordinate analysis on unweighted (considering presence/absence OTU) and weighted (community membership and abundance of OTUs) UniFrac distance matrices did not reveal any significant difference in the microbial communities between dogs with a diagnosis of IBD and those dogs with FRD neither in the duodenum nor colon (Fig. 3).

This was further confirmed with ANOSIM test ($P > 0.05$) as shown in Table 3. Similarly, ANOSIM did not reveal any significant differences pre- and post-treatment within each disease group (Table 3). No significant association was identified between microbial communities and the clinical disease severity (i.e., CIBDAI score) before treatment (ANOSIM $P > 0.05$).

To determine the differences in bacterial composition between the dogs with IBD and those dogs with FRD, an LEfSe was utilized. Several bacterial taxa were found to be enriched in the two disease groups. In the duodenum of dogs with IBD, Mycoplasmataceae, Microbacteriaceae and Unclassified_Rhizobiales were abundant at the family level (LDA scores: 3.659, 3.608 and 3.656, respectively), and one unclassified genus each of the families Neisseriaceae (LDA score: 4.156), Microbacteriaceae (LDA score: 3.659) and Rhizobiales (LDA score: 3.666) was abundant at the genus level. In dogs with FRD, the genus *Bilophila* (LDA score: 3.165) was abundant in the duodenal mucosa. Also, in dogs with FRD, the family Burkholderiaceae and the genera *Carnobacterium*, *Burkholderia*, Unclassified_Helicobacteraceae and Unclassified_Coriobacteriaceae (LDA scores: 3.176, 2.869, 2.978, 3.192 and 2.899, respectively) were found to be enriched within the colonic mucosa.

An LEfSe was also used to assess the mucosal microbial composition before and after treatment. Several bacterial taxa were identified to differ in their abundance either before or after treatment (Tables 4-7). In dogs with FRD, for example, the genera *Enterococcus* (LDA score: 3.633), *Corynebacterium* (LDA score: 3.989) and *Delftia* (LDA score: 4.010) were abundant in the duodenum before treatment, whereas *Comamonas* (LDA score: 3.419) was significantly abundant in the duodenum after treatment. In the colon of dogs with FRD, the genera *Carnobacterium* (LDA score: 3.624) and *Burkholderia* (LDA score: 3.472) were significantly abundant before treatment, and the genera *Bacteroides* (LDA score: 4.548), *Gemella* (LDA score: 3.497) and *Peptococcus* (LDA score: 3.337) were abundant after treatment.

The family Micrococcaceae (LDA score: 3.407) and an unclassified genus of the family Neisseriaceae (LDA score: 4.254) were found to be enriched within the duodenum of dogs with IBD before treatment. Only an unclassified genus of the family Bradyrhizobiaceae (LDA score: 4.219) reached significant abundance in the duodenum after treatment. The families Planococcaceae (LDA score: 3.161) and Oxalobacteraceae (LDA score: 3.200), and the genera *Citrobacter* (LDA score: 4.254), *Burkholderia* (LDA score: 3.884) and Unclassified Oxalobacteraceae (LDA score: 3.868) were significantly abundant in the colonic mucosa of dogs with IBD before treatment, whereas the genus *Bacteroides* (LDA score: 4.549) was abundant after treatment.

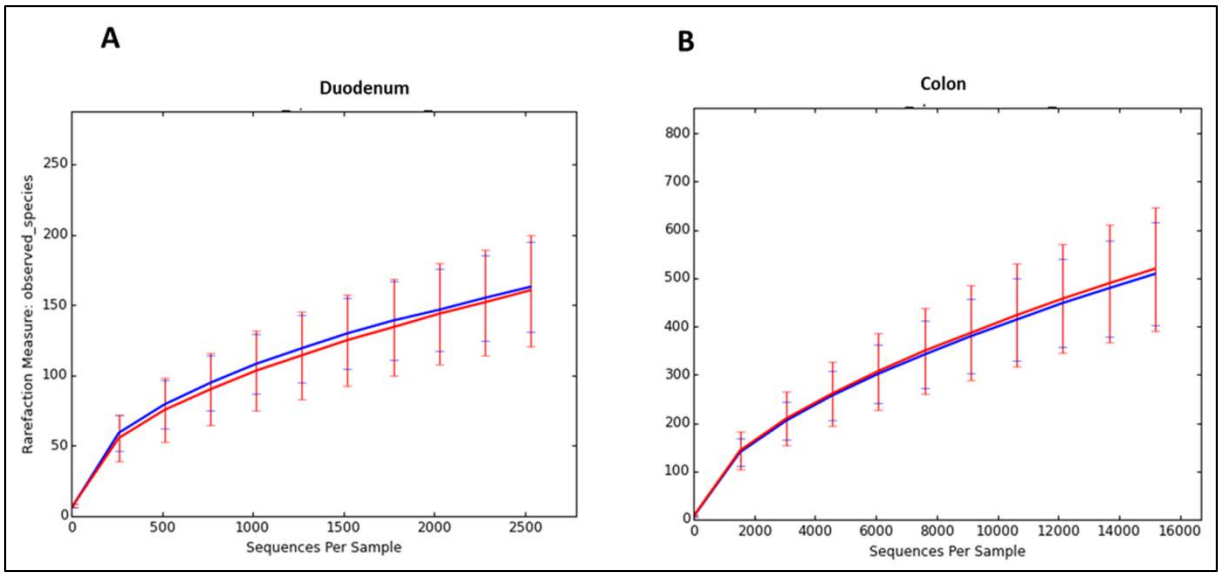

Figure 1. Rarefaction analysis of 16S rRNA gene sequences obtained from canine (A) duodenal and (B) colonic mucosa samples. Rarefaction depth was set at 15,170 (colon) and 2,530 (duodenum) sequences per sample. The lines (red = dogs with FRD; blue = dogs with IBD) represent the average of each group. The error bars represent the standard deviation.

Table 2. Summary of alpha diversity indices comparing dogs with IBD and FRD in colonic and duodenal samples.

	Median (Min -Max)						
Duodenum	**FRD-Pre**	**FRD-Post**	**IBD-Pre**	**IBD-Post**	**FRD vs IBD *P*-value***	***FRD* *Pre vs Post* *P*-value****	***IBD* *Pre vs Post* *P*-value****
Chao1	347(220-542)	352(170-773)	305(187-792)	273(248-434)	0.640	0.946	1
Observed OTU	153(103-250)	156(98-211)	168(127-230)	139(112-174)	0.480	0.893	0.129
Shannon	4.8(2.5-6.1)	5.1(2.6-6.1)	5.06(3.7-5.9)	4.62(4.03-5.43)	0.689	0.541	0.359
Colon	**FRD-Pre**	**FRD-Post**	**IBD-Pre**	**IBD-Post**	***FRD vs IBD* *P-value***	***FRD* *Pre vs Post* *P-value***	**IBD *Pre vs Post* *P-value***
Chao1	1275(714-1747)	1397(911-1892)	1193(554-2118)	1409(898-1730)	0.975	0.833	0.468
Observed OTU	511(340-741)	573(388-761)	531(316-651)	539(380-728)	0.850	0.570	0.2969
Shannon	4.9(2.4-6.3)	5.4(4.4-6.3)	5.1(2.5-5.8)	5.5(3.5-6)	0.874	0.052	0.9375

Rarefaction depth was set at 15,170 (colon) and 2,530 (duodenum) sequences/sample.
∗P values obtained by Mann–Whitney U test. P values < 0.05 considered as significant.
∗∗P values obtained by Wilcoxon signed-rank test. P values < 0.05 considered as significant.

DISCUSSION

To our knowledge, this is the first study to evaluate the intestinal mucosal microbiota of dogs with IBD or FRD both before and after treatment. This study did not reveal any differences in the overall species richness in dogs diagnosed with IBD and dogs with FRD. This finding could be attributed to the fact that both conditions may represent a different spectrum of the same disease, reflected by similar histologic inflammatory lesions (Day et al. 2008), and that can only be differentiated by their response to treatment (German, Hall and Day 2003; Simpson and Jergens 2011; Dandrieux 2016). It appears reasonable to assume that similar inflammatory histologic lesions might be associated with a similar effect on the mucosal microbiome. However, further studies are warranted to prove or disprove this hypothesis.

Analysis of the specific bacterial taxa in dogs with FRD and dogs with idiopathic IBD showed a differential abundance of mainly bacteria belonging to the phylum of Proteobacteria (e.g., *Bilophila* in the duodenum, *Burkholderia* and Unclassified Helicobacteraceae in the colon of FRD dogs; Unclassified Neisseriaceae and Unclassified Rhizobiales in the duodenum of IBD dogs). This finding agrees with recent studies revealing an increase of Proteobacteria in dogs with IBD (Suchodolski et al. 2010, 2012a; Minamoto et al. 2015). Proteobacteria have been shown to also belong to the most abundant phyla in the gastrointestinal tract of healthy dogs (Suchodolski, Camacho and Steiner 2008), where they are abundant especially in the small intestine and present at lower abundance in the colon (Suchodolski, Camacho and Steiner 2008; Schmitz and Suchodolski 2016). However, some members of this phylum have also been shown to have pathogenic characteristics. One variant of a novel family of Burkholderiales, for example, was associated with perianal Crohn's disease in humans (Sim et al. 2010). However, the association of pathogenic *Burkholderia* and canine chronic enteropathies has not been investigated.

Comparison of differentially abundant bacteria in dogs with FRD considering treatment status revealed the distinctive presence of bacteria in the duodenal mucosa before treatment. These bacteria belonged to the phyla Proteobacteria (*Delftia*), Actinobacteria (*Corynebacterium*) and Firmicutes (*Enterococcus*).

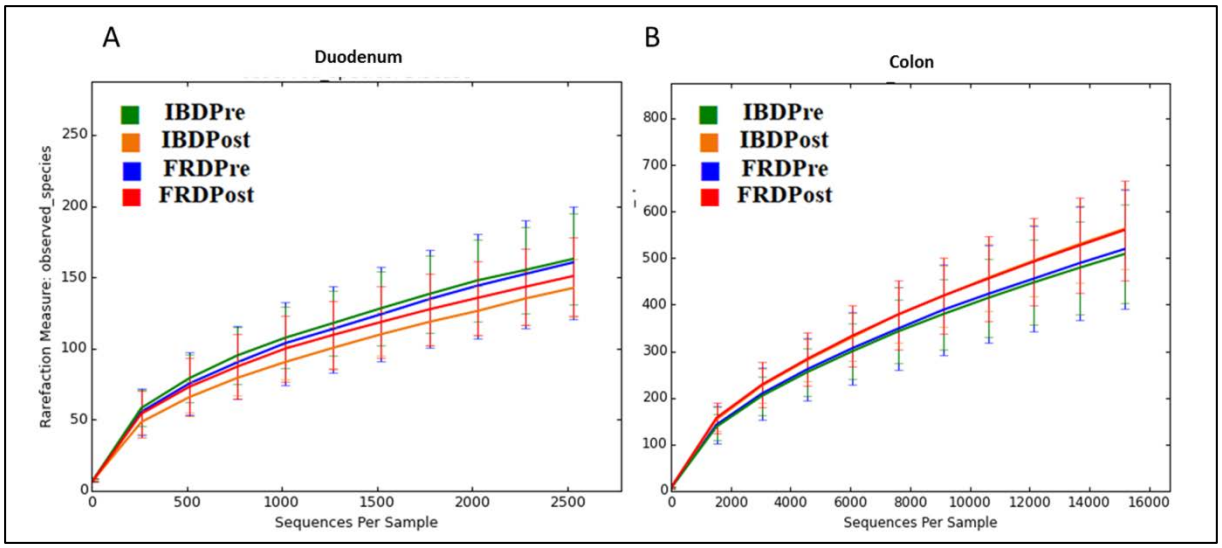

Figure 2. Rarefaction analysis of 16S rRNA gene sequences obtained from canine (A) duodenal and (B) colonic mucosa samples. The analysis was performed on a randomly selected subset of Table 2. Rarefaction depth was set at 15,170 (colon) and 2,530 (duodenum) sequences per sample. The lines represent the average of each group. The error bars represent the standard deviation.

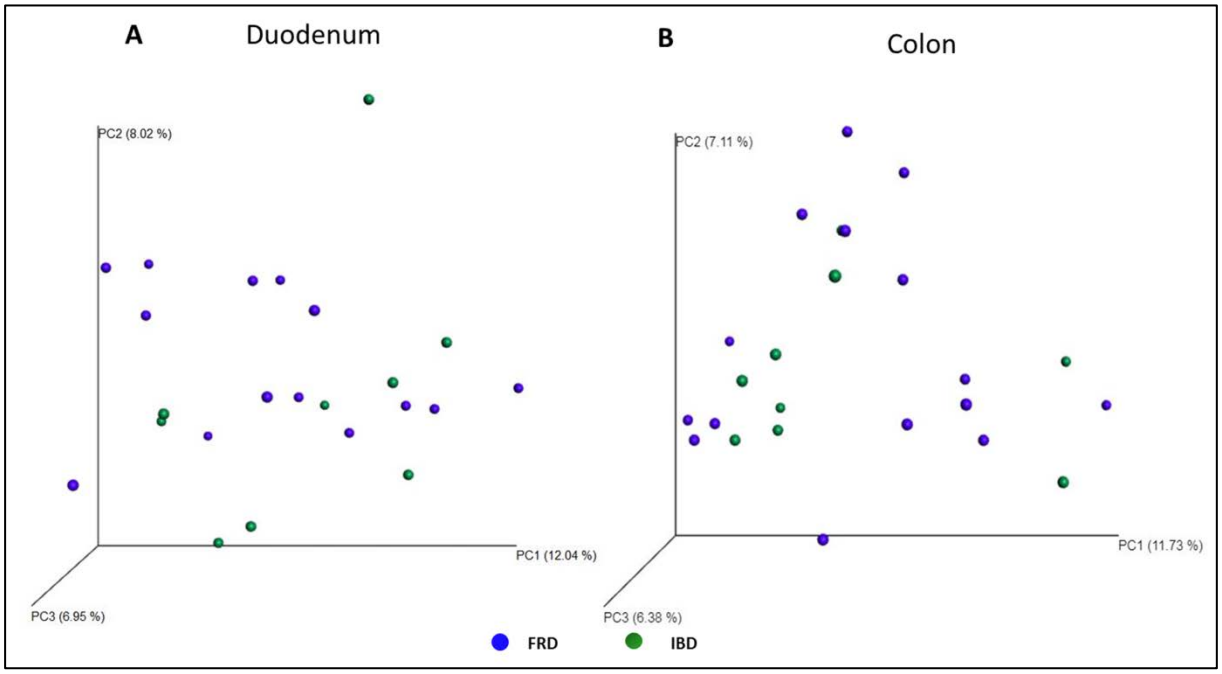

Figure 3. Three-dimensional principal coordinate analyses of unweighted uniFrac distances colored by disease in (A) duodenal and (B) colonic mucosal samples. Each dot represents the microbial composition of one dog. ANOSIM based on unweighted andweighted UniFrac distances did not showa significant difference between dogs diagnosed with FRD and dogs diagnosed with IBD in the duodenum and colon respectively (duodenum ANOSIM Punweighted = 0.694; colon ANOSIM Punweighted = 0.969; P values < 0.05 considered as significant).

The abundance of Proteobacteria before treatment is in line with the increase in Proteobacteria previously documented in dogs with IBD (Suchodolski et al. 2012a; Minamoto et al. 2015). The pathogenicity of particular strains of *Corynebacterium* has been known for many years (Dalal and Likhi 2008; Bernard 2012; Wagner et al. 2012) and a recent study has also reported *Corynebacterium* to be associated with primary sclerosing cholangitis with concomitant colonic disease (Bajer et al. 2017). Thus, the abundance of *Corynebacterium* before treatment in the current study might suggest involvement in the pathogenesis of chronic enteropathies. Certain *Enterococcus* strains are associated with beneficial effects and are used as probiotics (Kilpinen et al. 2015; Schmitz and Suchodolski 2016). In our study, *Enterococcus* was significantly abundant before treatment in the duodenum of dogs diagnosed with FRD. This finding is in contrast to a previous study in dogs with FRD that showed no significant change in the abundance of *Enterococcus* spp. with treatment and with the addition of a probiotic mixture (Sauter et al. 2006). This discrepancy might be explained by the different sampling methods, different methods to evaluate the microbiota or the difference in the study population or diet. One possible explanation for the differential abundance before treatment in the current study might be that *Enterococcus* with a higher abundance is more likely to modulate the inflammatory response and to keep the intestinal ecosystem in balance. Another consideration is the potential of *Enterococcus* to express virulence factors as has been shown in children with IBD (Golińska et al. 2013). Such virulence factors could potentially contribute to the inflammatory state and could also be associated with the pathogenesis of the disease. However, in our study both *Enterococcus* and *Corynebacterium* were only identified at the genus level and the specific strain was not analyzed due to an inadequate amount of residual DNA. Therefore, no conclusions can be drawn from our findings as to the functional relationship between higher abundance of *Enterococcus* or *Corynebacterium* and chronic intestinal inflammation. Future studies analyzing the specific strains of significantly abundant bacteria via quantitative real-time PCR are warranted.

In the colon of dogs with FRD, mainly bacteria of the phylum Firmicutes were differentially abundant both before and after treatment. However, one representative of the phylum Bacteroidetes – the genus *Bacteroides* – was also found to be enriched within the colonic mucosa after initiation of treatment. Since Bacteroidaceae have been detected to be more abundant in healthy dogs than in dogs with IBD (Suchodolski et al. 2012a; Minamoto et al. 2015), this finding might be associated with the effect of treatment and disease amelioration. This conclusion is also supported by the fact that certain *Bacteroides* strains have been recognized to be very beneficial to their host due to an important role in microbial degradation of carbohydrates (Flint et al. 2012) and bile acid deconjugation (Pavlidis et al. 2015). However, *Bacteroides* strains can also express virulence factors and promote

abscess formation via their polysaccharide capsule, and could potentially have negative effects on the host (Wexler 2007; Reis et al. 2014). Therefore, *Bacteroides* can be either protective or have virulent features, opening new avenues for further investigation of its role in the pathogenesis of chronic enteropathies in dogs, at best including the identification of specific strains.

Table 3. ANOSIM test based on weighted and unweighted UniFrac distances.

	Weighted		Unweighted	
	R value	*P*-value	R value	*P*-value
Duodenum				
FRD vs IBD	–0.0090	0.474	–0.0365	0.649
FRD pre vs FRD post	–0.0103	0.554	0.0318	0.233
IBD pre vs IBD post	–0.1020	0.947	0.0082	0.414
Colon				
FRD vs IBD	0.0399	0.264	–0.1192	0.969
FRD pre vs FRD post	0.0206	0.231	0.0338	0.181
IBD pre vs IBD post	0.0323	0.207	–0.0250	0.741

The ANOSIM test was used to determine if any groups of samples contained significantly different bacterial communities. R values close to 1 indicate high separation between groups. R values close to 0 indicate similarity between groups. P values < 0.05 considered as significant.

Table 4. LDA scores for association between treatment status and bacterial taxa in duodenal mucosal specimens from dogs diagnosed with IBD (n = 9).

Selected Taxa	LDA score (log 10)	Associated disease group
Phylum		
Tenericutes	3.245	IBD pre
Class		
Mollicutes	3.245	IBD pre
Order		
No differentially abundant features found		
Family		
Micrococcaceae	3.407	IBD pre
Genus		
Unclassified	4.085	IBD pre
Unclassified_Neisseriaceae	4.254	IBD pre
Unclassified_Bradyrhizobiaceae	4.219	IBD post

A LDA score >2.0 is considered significant.
pre, status before treatment; post, status after treatment.

Table 5. LDA scores for association between treatment status and bacterial taxa in colonic mucosal specimens from dogs diagnosed with IBD (n = 9).

Selected Taxa	LDA Score (log 10)	Associated disease group
Phylum		
Bacteroidetes	5.026	IBD post
Class		
Bacteroidia	5.024	IBD post
Order		
Bacteroidales	5.025	IBD post
Family		
Planococcaceae	3.161	IBD pre
Oxalobacteraceae	3.200	IBD pre
Bacteroidaceae	4.542	IBD post
Genus		
Unclassified_Oxalobacteraceae	3.868	IBD pre
Citrobacter	4.254	IBD pre
Burkholderia	3.884	IBD pre
Bacteroides	4.549	IBD post

A LDA score >2.0 is considered significant.
pre, status before treatment; post, status after treatment.

Evaluation for possible differences in the bacterial taxa in dogs with IBD depending on treatment status revealed an increased abundance of mainly members of the phylum Proteobacteria (unclassified genus of the family Neisseriaceae in the duodenum; unclassified genus of the family Oxalobacteraceae, and the genera *Citrobacter* and *Burkholderia* in the colon) before treatment. Further, one representative of the phylum Firmicutes (family Planococcaceae in the colon) was found at a higher abundance before treatment. To the authors' knowledge, no specific pathogenic characteristics have been reported for this particular family found in our study. Therefore, the relevance of our finding remains unclear.

Only an unclassified genus of the family Bradyrhizobiaceae was found to be enriched in the duodenum of dogs with IBD post-treatment. Again, to the authors' knowledge, no pathogenic or constitutional trait has been reported for this bacterium, leaving the relevance of this finding unclear. An important finding, however, is that just as in dogs with FRD *Bacteroides* also reached significant abundance in the colon of dogs with IBD after treatment. This increase of *Bacteroides* in both disease groups after treatment possibly suggests disease amelioration, and therefore this bacterium could potentially be used as a respective marker of treatment response.

Table 6. LDA scores for association between treatment status and bacterial taxa in duodenal mucosal specimens from dogs diagnosed with FRD (n = 13).

Selected Taxa	LDA Score (log 10)	Associated disease group
Phylum		
No differentially abundant features found		
Class		
No differentially abundant features found		
Order		
No differentially abundant features found		
Family		
Peptostreptococcaceae	3.694	FRD pre
Enterococcaceae	3.789	FRD pre
Oxalobacteraceae	3.766	FRD pre
Comamonadaceae	4.280	FRD pre
Bacillaceae	3.848	FRD pre
Corynebacteriaceae	3.952	FRD pre
Genus		
Unclassified_Peptostreptococcaceae	3.664	FRD pre
Enterococcus	3.633	FRD pre
Unclassified_Coriobacteriaceae	3.855	FRD pre
Corynebacterium	3.989	FRD pre
Delftia	4.010	FRD pre
Comamonas	3.419	FRD post

A LDA score >2.0 is considered significant.
pre, status before treatment; post, status after treatment.

The absence of a group of healthy control dogs poses a major limitation of this study. However, in order to follow a standardized study protocol, endoscopies involving general anesthesia would have been necessary before treatment and potentially also after a standardized treatment in these dogs. Because of this invasiveness and the fact that several studies evaluating the intestinal microbiota in healthy dogs have already been published (Suchodolski, Camacho and Steiner 2008; Handl et al. 2011), performing repeated gastrointestinal endoscopies under general anesthesia in healthy dogs was considered unethical. Examination of the microbiota in fecal samples of healthy controls both before and after treatment with the study's diet could have been a less invasive alternative to endoscopy. However, a study by one of the authors (JS) has revealed bacterial diversity along the different sections of the canine intestine (Suchodolski, Camacho and Steiner 2008). Therefore, a direct comparison of the fecal microbiota of healthy dogs and, for example, the duodenal mucosal microbiota of diseased dogs would not have been meaningful. The mucosal samples of this study were purposefully retrieved as part of a larger study, and the intestinal microbiota was analyzed retrospectively for this specific study.

Unfortunately, residual fecal samples of the dogs diagnosed with IBD and FRD were not available for an additional analysis of the fecal microbiota in the current study. Moreover, in the authors' opinion, mucosal samples are superior to fecal samples in assessing the true intestinal microbiota.

Table 7. LDA scores for association between treatment status and bacterial taxa in colonic mucosal specimens from dogs diagnosed with FRD (n = 14).

Selected Taxa	LDA Score (log 10)	Associated disease group
Phylum		
No differentially abundant features found		
Class		
Bacteroidia	4.821	FRD post
Order		
Gemellales	2.911	FRD post
Bacteroidales	4.491	FRD post
Family		
Burkholderiaceae	3.132	FRD pre
Bacteroidaceae	4.512	FRD post
Peptococcaceae	3.342	FRD post
Gemellaceae	3.517	FRD post
Genus		
Burkholderia	3.472	FRD pre
Carnobacterium	3.624	FRD pre
Gemella	3.497	FRD post
Peptococcus	3.337	FRD post
Bacteroides	4.548	FRD post

A LDA score >2.0 is considered significant.
pre, status before treatment; post, status after treatment.

Genomic DNA was extracted using the Mo Bio PowerSoil® DNA Isolation Kit, which is used in the Human Microbiome Project and is currently recommended for the extraction of DNA. The use of this method yielded the currently most effective DNA extraction (Wagner Mackenzie, Waite and Taylor 2015) and kept the possible influence of sample preparation on this study's results to a minimum.

The different time scales for the follow-up endoscopies in the two disease groups could have possibly influenced the results. However, as glucocorticoids were not instituted until there was no significant clinical improvement on the elimination diet only on day 14, those dogs had to be given more time to respond to treatment. In general, FRD dogs are considered to respond faster to appropriate treatment, whereas dogs requiring immunosuppressants usually take longer to improve clinically. The different time scales were adopted from

previous studies (Allenspach et al. 2007; Burgener et al. 2008; Dumusc et al. 2014) and are considered more representative of a similarly controlled disease status.

As most dogs diagnosed with FRD show a significant clinical response to a dietary trial within 14 days (Marks, Laflamme and McAloose 2002; Allenspach et al. 2007; Gaschen and Merchant 2011; Allenspach, Culverwell and Chan 2016), the elimination trial was limited to that period of time. Furthermore, this short period provided for good owner compliance. Dietary modification is also an inherent part of treatment in dogs with IBD. Thus, it is possible that some dogs with IBD could have initially improved on the diet alone and could have been erroneously grouped in the FRD group. Yet, in the authors' experience, dogs diagnosed with IBD generally only show minor improvement on elimination diet and would have probably not been assessed as significant improvement, much less as clinical remission.

Another limitation of our study is the small number of dogs included and the fact that endoscopic biopsies were only obtained from the duodenum and colon. A larger study cohort of dogs and inclusion of additional biopsy sites (particularly the ileum) might have yielded more obvious differences in the mucosal microbial composition. Another shortcoming of our study is that the possibility of a continuing effect of previous medications (e.g., antibiotic treatment) on the mucosal microbiota cannot be definitively excluded, although all efforts were made to minimize such an effect by including only dogs not given an antibiotic 2 weeks prior to enrollment in the study. According to studies in human medicine, a 4-week wash-out period (Dethlefsen et al. 2008; Langdon, Crook and Dantas 2016) may be more appropriate than a 2-week period and may have resulted in finding a more restored and original intestinal microbial composition. A study in veterinary medicine by one of the authors (JS), however, showed that the intestinal microbiota of dogs that received metronidazole returned to its original composition within 14 days after cessation of therapy in the majority of dogs (Olson et al. 2015). Until now, there are only limited studies examining the effect of glucocorticoids on the intestinal microbial composition. One study revealed no effect of prednisolone on the fecal microbiota of dogs (Igarashi et al. 2014) and to the authors' knowledge, there are no studies evaluating the influence of budesonide on the intestinal microbiota. The authors did not expect a major impact of budesonide on the microbial composition, and therefore, the one dog receiving budesonide was still included in the analysis.

The possibility of a long-term effect of the previous diet and prebiotics or probiotics given prior to the study can also not be entirely ruled out. Studies in both human and veterinary medicine have shown that the diet, in particular macronutrients, influences the intestinal microbial composition (Hang et al. 2012; Conlon and Bird 2014; Xu and Knight 2015; Herstad et al. 2017; Li et al. 2017). However, the diets used in those studies were diets with pronounced alterations in the composition of macronutrients, which do not conform to

commonly used diets. Moreover, a recent study showed that alpha diversity did not correlate with fat or protein intake in a population of dogs with IBD and control dogs (Vázquez-Baeza et al. 2016), concluding that the disease effect is stronger than the diet's effect on the microbial composition. It has been recognized that obese individuals harbor a different intestinal microbiota with especially a decreased abundance of Bacteroides and an increased abundance of Firmicutes, which leads to an increased capacity to harvest energy (Ley et al. 2005; Turnbaugh et al. 2006, 2009). Thus, the body condition score (BCS) of dogs could have affected the results of the current study. However, 16 dogs were assigned a BCS between 4 and 6 out of 9 at their initial visit, representing a similar BCS for the majority of dogs. Furthermore, the study by Vázquez-Baeza et al. also revealed no correlation of the BCS and alpha diversity in the enrolled dogs. Therefore, the authors consider the influence of the BCS on this study's results negligible. An influence of lifestyle (e.g., smoking, exercising, stress) on the intestinal microbiota has been shown in humans (Conlon and Bird 2014), with, for example, a higher diversity of the intestinal microbes in extreme athletes (Clarke et al. 2014). Although similar effects can be assumed in athletic or working dogs and dogs living in a smoking household, these effects are again only expected with extreme lifestyles, not reflecting the average companion dog. One study reported both age and breed, respectively size, to have an impact on the intestinal microbial composition in dogs (Simpson et al. 2002), whereas another study did not find a correlation between age and the alpha diversity in dogs (Vázquez-Baeza et al. 2016). More studies are needed to evaluate the true impact of these factors on the intestinal microbiota. All of the aforementioned possible influences could not be fully excluded in this study, as the dogs' characteristics and environment could not be standardized in the case of a clinical trial. However, in the authors' opinion, the diversity of dogs enrolled in this study represents a more realistic and therefore meaningful patient population.

In conclusion, this study is the first to assess the intestinal mucosal microbiota in dogs with IBD or FRD before and after treatment. Some differences in individual bacterial taxa were identified both between disease groups and in relation to the treatment status. The relevance of these bacterial groups in the pathogenesis of IBD and FRD requires further research. However, our results suggest that Bacteroides might be a possible marker of successful response to treatment. Larger scale studies are warranted to confirm our findings and to shed more light on the functional consequences of the changes in the intestinal microbial composition shown in this study. Evaluation of their role in the pathophysiology of IBD and FRD and of their potential therapeutic benefit warrants further study.

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REFERENCES

Allenspach K, Culverwell C, Chan D. Long-term outcome in dogs with chronic enteropathies: 203 cases. *Vet Rec* 2016;178:368.

Allenspach K, House A, Smith K et al. Evaluation of mucosal bacteria and histopathology, clinical disease activity and expression of Toll-like receptors in German shepherd dogs with chronic enteropathies. *Vet Microbiol* 2010;146:326–35.

Allenspach K, Wieland B, Gröne A et al. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. *J Vet Intern Med* 2007;21:700–8.

Bajer L, Kverka M, Kostovcik M et al. Distinct gut microbiota profiles in patients with primary sclerosing cholangitis and ulcerative colitis. *World J Gastroenterol* 2017;23:4548–58.

Bell ET, Suchodolski JS, Isaiah A et al. Faecal microbiota of cats with insulin-treated diabetes mellitus. *PLoS One* 2014;9:e108729.

Bernard K. The genus *Corynebacterium* and other medically relevant coryneform-like bacteria. *J Clin Microbiol* 2012;50:3152–8.

Burgener IA, König A, Allenspach K et al. Upregulation of Toll-like receptors in chronic enteropathies in dogs. *J Vet Intern Med* 2008;22:553–60.

Caporaso JG, Kuczynski J, Stombaugh J et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010;7:335–6.

Cassmann E, White R, Atherly T et al. Alterations of the ileal and colonic mucosal microbiota in canine chronic enteropathies. *PLoS One* 2016;11:e0147321.

Clarke SF, Murphy EF, Sullivan O et al. Exercise and associated dietary extremes impact on gut microbial diversity. *Gut* 2014;63:1913.

Conlon MA, Bird AR. The impact of diet and lifestyle on gut microbiota and human health. *Nutrients* 2014;7:17–44.

Dalal A, Likhi R. *Corynebacterium minutissimum* bacteremia and meningitis: a case report and review of literature. *J Infect* 2008;56:77–79.

Dandrieux JR. Inflammatory bowel disease versus chronic enteropathy in dogs: are they one and the same? *J Small Anim Pract* 2016;57:589–99.

Day MJ, Bilzer T, Mansell J et al. Histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from the dog and cat: a report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group. *J Comp Pathol* 2008;138(Suppl 1): S1–43.

DeSantis TZ, Hugenholtz P, Larsen N et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microb* 2006;72: 5069–72.

Dethlefsen L, Huse S, Sogin ML et al. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol* 2008;6:e280.

Dumusc SD, Ontsouka EC, Schnyder M et al. Cyclooxygenase-2 and 5-lipoxygenase in dogs with chronic enteropathies. *J Vet Intern Med* 2014;28:1684–91.

Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;26:2460–1.

Flint HJ, Scott KP, Duncan SH et al. Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* 2012;3:289–306.

Garcia-Mazcorro JF, Dowd SE, Poulsen J et al. Abundance and short-term temporal variability of fecal microbiota in healthy dogs. *Microbiology Open* 2012;1:340–7.

Gaschen FP, Merchant SR. Adverse food reactions in dogs and cats. *Vet Clin North Am-Small* 2011;41:361–79.

German AJ, Hall EJ, Day MJ. Chronic intestinal inflammation and intestinal disease in dogs. *J Vet Intern Med* 2003;17:8–20.

Golińska E, Tomusiak A, Gosiewski T et al. Virulence factors of *Enterococcus* strains isolated from patients with inflammatory bowel disease. *World J Gastroenterol* 2013;19:3562–72.

Guard BC, Suchodolski JS. HORSE SPECIES SYMPOSIUM: Canine intestinal microbiology and metagenomics: From phylogeny to function. *J Anim Sci* 2016;94:2247–61.

Hall EJ, German AJ. Diseases of the small intestine. In: Ettinger SJ, Feldman EC (eds). *Textbook of Veterinary Internal Medicine*, Vol. 2. Missouri, USA: Saunders Elsevier, St. Louis, 2010, 1560–6.

Handl S, Dowd SE, Garcia-Mazcorro JF et al. Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. *FEMS Microbiol Ecol* 2011;76:301–10.

Hang I, Rinttila T, Zentek J et al. Effect of high contents of dietary animal-derived protein or carbohydrates on canine faecal microbiota. *BMC Vet Res* 2012;8:90.

Herstad KMV, Gajardo K, Bakke AM et al. A diet change from dry food to beef induces reversible changes on the faecal microbiota in healthy, adult client-owned dogs. *BMC Vet Res* 2017;13:147.

Igarashi H, Maeda S, Ohno K et al. Effect of oral administration of metronidazole or prednisolone on fecal microbiota in dogs. *PLoS One* 2014;9:e107909.

Jergens A, Moore F, Haynes J et al. Idiopathic inflammatory bowel disease in dogs and cats: 84 cases (1987-1990). *J Am Vet Med Assoc* 1992;201:1603–8.

Jergens AE, Schreiner CA, Frank DE et al. A scoring index for disease activity in canine inflammatory bowel disease. *J Vet Intern Med* 2003;17:291–7.

Kathrani A, Lee H, White C et al. Association between nucleotide oligomerisation domain two (Nod2) gene polymorphisms and canine inflammatory bowel disease. *Vet Immunol Immunop* 2014;161:32–41.

Kilpinen S, Rantala M, Spillmann T et al. Oral tylosin administration is associated with an increase of faecal enterococci and lactic acid bacteria in dogs with tylosin-responsive diarrhoea. *Vet J* 2015;205:369–74.

Kilpinen S, Spillmann T, Syrja P et al. Effect of tylosin on dogs with suspected tylosin-responsive diarrhea: a placebo-controlled, randomized, double-blinded, prospective clinical trial. *Acta Vet Scand* 2011;53:26.

Langdon A, Crook N, Dantas G. The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation. *Genome Med* 2016;8: 39.

Ley RE, Backhed F, Turnbaugh P et al. Obesity alters gut microbial ecology. *P Natl Acad Sci USA* 2005;102:11070–5.

Li Q, Lauber CL, Czarnecki-Maulden G et al. Effects of the dietary protein and carbohydrate ratio on gut microbiomes in dogs of different body conditions. *mBio* 2017;8:e01703–01716.

Mandigers PJ, Biourge V, van den Ingh TS et al. A randomized, open-label, positively-controlled field trial of a hydrolyzed protein diet in dogs with chronic small bowel enteropathy. *J Vet Intern Med* 2010;24:1350–7.

Marks S, Laflamme DP, McAloose D. Dietary trial using a commercial hypoallergenic diet containing hydrolyzed protein for dogs with inflammatory bowel disease. *Vet Ther* 2002;3:109–18.

Minamoto Y, Otoni CC, Steelman SM et al. Alteration of the fecal microbiota and serum metabolite profiles in dogs with idiopathic inflammatory bowel disease. *Gut Microbes* 2015;6:33– 47.

Olson E, Honneffer JB, Waddle M et al. Evaluation of the effects of a 2 week treatment with metronidazole on the fecal microbiome of healthy dogs (abstr.). *J Vet Intern Med* 2015;29:1184.

Pavlidis P, Powell N, Vincent RP et al. Systematic review: bile acids and intestinal inflammation-luminal aggressors or regulators of mucosal defence? *Aliment Pharm Ther* 2015;42:802–17.

Reis AC, Silva JO, Laranjeira BJ et al. Virulence factors and biofilm production by isolates of Bacteroides fragilis recovered from dog intestinal tracts. *Braz J Microbiol* 2014;45:647–50.

Sauter SN, Benyacoub J, Allenspach K et al. Effects of probiotic bacteria in dogs with food responsive diarrhoea treated with an elimination diet. *J Anim Physiol An N* 2006;90:269–77.

Schmitz S, Suchodolski J. Understanding the canine intestinal microbiota and its modification by pro-, pre- and synbiotics - what is the evidence? *Vet Med Sci* 2016;2:71–94.

Segata N, Izard J, Waldron L et al. Metagenomic biomarker discovery and explanation. *Genome Biol* 2011;12:R60.

Sim WH, Wagner J, Cameron DJ et al. Novel Burkholderiales 23S rRNA genes identified in ileal biopsy samples from children: preliminary evidence that a subtype is associated with perianal Crohn's disease. *J Clin Microbiol* 2010;48:1939–42.

Simpson JM, Martineau B, Jones WE et al. Characterization of fecal bacterial populations in canines: effects of age, breed and dietary fiber. *Microb Ecol* 2002;44:186–97.

Simpson KW, Jergens AE. Pitfalls and progress in the diagnosis and management of canine inflammatory bowel disease. *Vet Clin N Am Small* 2011;41:381–98.

Suchodolski JS, Camacho J, Steiner JM. Analysis of bacterial diversity in the canine duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis. *FEMS Microbiol Ecol* 2008;66:567–78.

Suchodolski JS, Dowd SE, Wilke V et al. 16S rRNA gene pyrosequencing reveals bacterial dysbiosis in the duodenum of dogs with idiopathic inflammatory bowel disease. *PLoS One* 2012a;7:e39333.

Suchodolski JS, Markel ME, Garcia-Mazcorro JF et al. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. *PLoS One* 2012b;7:e51907.

Suchodolski JS, Xenoulis PG, Paddock CG et al. Molecular analysis of the bacterial microbiota in duodenal biopsies from dogs with idiopathic inflammatory bowel disease. *Vet Microbiol* 2010;142:394–400.

Turnbaugh PJ, Ley RE, Mahowald MA et al. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006;444:1027–131.

Turnbaugh PJ, Hamady M, Yatsunenko T et al. A core gut microbiome in obese and lean twins. *Nature* 2009;457:480–4.

Vázquez-Baeza Y, Hyde ER, Suchodolski JS et al. Dog and human inflammatory bowel disease rely on overlapping yet distinct dysbiosis networks. *Nat Microbiol* 2016;1:16177.

Wagner KS, White JM, Lucenko I et al. Diphtheria in the postepidemic period, Europe, 2000–2009. *Emerg Infect Dis* 2012;18:217–25.

Wagner Mackenzie B, Waite DW, Taylor MW. Evaluating variation in human gut microbiota profiles due to DNA extraction method and inter-subject differences. *Front Microbiol* 2015;6:130.

Westermarck E, Skrzypczak T, Harmoinen J et al. Tylosin-responsive chronic diarrhea in dogs. *J Vet Intern Med* 2005;19:177–86.

Wexler HM. Bacteroides: the good, the bad, and the nitty-gritty. *Clin Microbiol Rev* 2007;20:593–621.

Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 2007;448:427–34.

Xu Z, Knight R. Dietary effects on human gut microbiome diversity. *Br J Nutr* 2015;113(Suppl):S1–5.

3.2 Comparison of the Systemic Phospholipid Profile in Dogs Diagnosed with Idiopathic Inflammatory Bowel Disease or Food-Responsive Diarrhea before and after Treatment

I contributed to this second study by being responsible for the project administration including all management and coordination responsibilities for pursuing this project. I was further responsible for interpretation of the results in view of the clinical aspects and the relevance of the findings for the general veterinary and the greater scientific community. I gathered and analyzed the data acquired by hydrophilic interaction liquid chromatography (HILIC) performed by my co-authors, and I wrote this manuscript.

Comparison of the systemic phospholipid profile in dogs diagnosed with idiopathic inflammatory bowel disease or food-responsive diarrhea before and after treatment

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Abstract

Background

Inflammatory bowel disease (IBD) and food-responsive diarrhea (FRD) are common chronic enteropathies in dogs, of which the exact pathogenesis has not been fully understood. In people dyslipidemia has been reported in patients with IBD, and potential therapeutic benefits of polyunsaturated fatty acids (PUFA) in the treatment of IBD have been investigated. Studies on the phospholipid profile in dogs with IBD and FRD are still lacking.

Aim

To investigate the systemic phospholipid profile of dogs with IBD or FRD and to evaluate possible differences in phospholipids before and after treatment.

Methods

The phospholipids in whole blood and EDTA plasma of 32 dogs diagnosed with either IBD (n=16) or FRD (n=16) were analyzed by hydrophilic interaction liquid chromatography (HILIC) prior to and after initiation of treatment, which included an elimination diet enriched with PUFAs.

Results

A clear separation of the phospholipids between whole blood and plasma was demonstrated on principal component analysis plots. In addition to the type of specimen, treatment and disease severity were the most significant factors determining the variance of the phospholipid profile. An increase in lysolipids was observed after treatment. The phosphatidylcholine (PC) species changed from PC 38:4 before treatment to mainly lysophosphatidylcholine 18:0 after treatment. Furthermore, several differences in the abundance of individual phospholipids were identified between dogs with IBD and dogs with FRD and between treatment statuses using random forest analysis.

Conclusion

Significant variances were identified in the phospholipid profiles of dogs with IBD and FRD. These were particularly determined by type of specimen used, disease severity and treatment status. After treatment, a shift of phospholipid species towards lysophosphatidylcholine 18:0 was observed. Future studies should further investigate the role of lipids in the pathophysiology of IBD and FRD as well as their potential therapeutic benefits.

Introduction

Chronic inflammatory enteropathies (CIE) are a group of common disorders in dogs, which are categorized based on the patient's response to treatment as either food-responsive diarrhea (FRD), antibiotic-responsive diarrhea (ARD), or idiopathic inflammatory bowel disease (IBD) [1-4]. Dogs with FRD will show a complete clinical response after dietary modification to a novel source of protein and carbohydrates or to a commercially available hydrolyzed protein diet [5,6], whereas dogs with ARD require the use of antibiotic treatment, for example with tylosin, in addition to dietary management for clinical signs of gastrointestinal disease to resolve [7-9]. Idiopathic IBD is defined as chronic gastrointestinal signs of a complex pathogenesis, histologic confirmation of intestinal inflammation, and the necessity for anti-inflammatory and / or immunosuppressive treatment [2,4,7,10]. To date, the etiopathogenesis of CIE, in particular of idiopathic IBD, has not been fully unraveled. However, the current state of knowledge strengthens the notion that a combination of a genetic susceptibility [11-15], dietary and environmental factors, the intestinal microbiota, and an exaggerated immune response contribute to the development of idiopathic IBD in dogs [16-20]. This complexity involving the pathogenesis of IBD urgently asks for potential novel treatment strategies in addition to the currently used stepwise treatment approach of dietary modification, antibiotic trials, and immunosuppressive treatment [2,3]. Novel approaches, including beneficial alterations in the intestinal microbiota through the administration of probiotics and / or prebiotics [21-26] or fecal microbial transplants [27-29], have recently attracted great attention and warrant further research to fully elucidate their therapeutic potential or benefit.

In human medicine, alterations in lipid profiles and lipid homeostasis have been reported with several diseases, including metabolic syndrome [30], diabetes mellitus [30,31], myocardial infarction [31], Alzheimer's disease [32,33], and cancer [34]. Likewise, dyslipidemia has been detected in patients diagnosed with IBD [35-37]. Similar to the findings in humans, altered lipid profiles have been recognized in dogs diagnosed with idiopathic hyperlipidemia [38,39], diabetes mellitus [40], parvoviral infection [41], cancer [42], renal disease [43,44], or systemic infections [45]. Besides possibly contributing to the pathogenesis of those diseases, several studies have demonstrated lipids, in particular polyunsaturated fatty acids (PUFA), to also have immunomodulatory, anti-inflammatory, and potentially other beneficial effects both in human [46-48] and in veterinary patients [49,50]. These characteristics of PUFAs appear to be very promising from a therapeutic and even a preventative [51] perspective. However, there is currently only very limited data suggesting that dietary supplementation with PUFAs yields a clinical benefit in humans with IBD [36,47,48]. In veterinary medicine, only two studies have investigated the effect of supplemental PUFAs in dogs diagnosed with CIE. Those studies indicate that adding PUFAs to the diet might modify cholesterol homeostasis

[52] and also modulate the expression of genes affecting intestinal fatty acid uptake [53], both of which appear to be beneficial in dogs with CIE.

Phospholipids are amphiphilic lipids and represent fundamental components of biological membranes, where they are organized as lipid bilayers [54,55]. Due to their essential abundance in cell membranes their fatty acid composition has a major influence on membrane quality and phospholipids further serve as sources of fatty acids, including PUFAs, lipid mediators and molecules of cell signaling [55,56]. Thus, phospholipids and their membrane composition have great influence on health [55] and, similar to PUFAs, they have been reported to have beneficial effects in several diseases in humans [54,57-61].

To the authors' knowledge, the systemic phospholipid profiles have not been reported in dogs with CIE (neither before nor after initiation of treatment). Therefore, the objectives of this study were (1) to compare the phospholipid profiles between dogs with idiopathic IBD and dogs diagnosed with FRD, and (2) to evaluate the effect of treatment including dietary supplementation with PUFAs on the phospholipid composition in dogs with CIE by comparing the phospholipid profiles before and after induction therapy. It was hypothesized (1) that the phospholipid profiles differ between the two disease categories, and (2) that the phospholipid profiles also differ within each disease group depending upon the treatment status.

Materials and Methods

Animals and study protocol

Stored whole blood and plasma samples of a previously reported study on canine chronic enteropathies by one of the authors (Iwan A. Burgener) [11,62] were used for the current investigation. In that original study, dogs with chronic gastrointestinal signs, in the form of diarrhea with or without vomiting or weight loss for at least six weeks, were prospectively enrolled between December 2006 and November 2008. Additional inclusion criteria comprised the absence of an identifiable underlying disorder, histopathologic evidence of intestinal inflammation, and no treatment with antibiotics, corticosteroids, antisecretory medications, or combinations of these for at least two weeks prior to enrollment of dogs into the study. As most dogs had already received dietary modifications prior to referral, previous dietary trials did not preclude a dog's participation in this project. To exclude possible underlying disorders, a complete blood count, serum biochemistry profile, urinalysis, measurement of serum canine trypsin-like-immunoreactivity (cTLI), serum cobalamin and folate concentrations, adrenocorticotropic hormone stimulation test, parasitic and bacterial fecal examination (including Clostridium spp, Campylobacter spp and Salmonella spp), abdominal ultrasonography, and endoscopy of the gastrointestinal tract were performed in all

dogs. The specific canine pancreatic lipase test was not readily available in Europe between 2006 and 2008. Thus, a diagnosis of pancreatitis was ruled out based on a normal serum amylase and lipase activity, a normal serum cTLI concentration, and absence of abdominal ultrasound findings consistent with pancreatitis. All dogs were treated with an antiparasitic (fenbendazole 50 mg/kg p.o. SID for 5 days) irrespective of the results of the fecal parasite examination. Further, the body condition score (BCS) was recorded in the majority of dogs [63]. As the original sample collection was performed by Iwan A. Burgener at the Small Animal Teaching Hospital of the Vetsuisse Faculty, University of Bern, Switzerland, the design of the study was reviewed and approved by the Cantonal Committee of Animal Experimentation, Bern, Switzerland (permit number BE 118/05), and all owners gave written consent prior to inclusion of the dog in the study.

All dogs were assigned a clinical disease severity score (canine IBD activity index [CIBDAI] [64]) both prior to and after initiation of treatment. Each dog in the study was further classified as having signs of either predominantly small intestinal or large intestinal disease, or a combination of both. A gastroduodenoscopy and colonoscopy were performed in each dog enrolled in the study except for four dogs with severe hypoalbuminemia due to protein-losing enteropathy (PLE). In these dogs the preparatory 36-hour fast necessary for colonoscopy was considered potentially harmful, thus the endoscopic examination was limited to a gastroduodenoscopy.

After completion of this standard diagnostic work-up, including gastrointestinal endoscopy with collection of tissue biopsies, all dogs were fed a standardized elimination diet for 14 days. The study diet was a dry single protein diet based on codfish and rice only, with codfish being a novel source of protein for all dogs enrolled in the study. This elimination diet was specially produced for the study (Biomill SA, Granges-Marnand, Switzerland) and was enriched with PUFAs, yielding a concentration of omega-3 PUFAs of 1% and a concentration of omega-6 PUFAs of 3.5% (S1-S3 Files). The adequacy of the diet's nutritional composition was calculated by a veterinary nutritionist. Owners received detailed instructions on the concept of an elimination diet, strictly prohibiting table scraps and treats other than the prescribed diet. If clinical signs improved significantly or resolved within the first 14 days of feeding the study diet, dogs were assigned to the food-responsive (FRD) group. Dogs that did not improve clinically on the elimination diet were additionally treated with prednisolone (1 mg/kg p.o. BID) for 14 days followed by a slow tapering of the dose. These dogs were allocated to the idiopathic IBD / steroid-responsive disease group. Dogs that did not respond to prednisolone further received cyclosporine (5 mg/kg p.o. SID) or other immunosuppressants (e.g., budesonide 3 mg/m² p.o. SID).

The clinical evaluation after initiation of treatment consisted of a re-evaluation of the CIBDAI score in all dogs and a follow-up gastrointestinal endoscopy in the majority of dogs. The FRD group of dogs was reassessed four weeks after starting the elimination diet, whereas the IBD group of dogs was re-evaluated at 10 weeks after starting treatment with prednisolone.

Gastrointestinal endoscopy and histopathologic evaluation

Details on the endoscopic and the histopathologic evaluation have been published elsewhere [11]. Briefly, mucosal biopsy specimens were retrieved from the duodenum (approximately 10 cm proximal to the caudal duodenal flexure) and the colon (the middle portion of the descending colon) or from areas with any obvious lesions. Samples were placed in 4% neutral-buffered formalin for 48 hours before being embedded in paraffin and subsequently prepared for histopathologic evaluation.

The endoscopic biopsies were examined histologically by a board-certified pathologist blinded to clinicopathological findings, the number of endoscopy, diagnosis, and treatment. The pathologist assigned a histologic lesion score reflecting the degree of inflammation and cellular infiltration [65].

Blood sample collection and analysis of the phospholipid profile

During the initial diagnostic work-up and the reassessment of the dogs after initiation of treatment, whole blood and EDTA-plasma samples were collected and immediately stored frozen at -20 °C until lipid extraction.

For analysis of the phospholipid profile, 20-200 µL of sample (whole blood or plasma) was mixed with 3.75 volumes of mass spectrometry (MS) grade chloroform:methanol (1:2 [v/v]). After 30 minutes, samples were centrifuged for 5 minutes at 2000×g to remove protein precipitate. The supernatant was then transferred to glass autosampler vials for immediate analysis. Hydrophilic interaction liquid chromatography (HILIC) of polar lipid classes was performed on the extracts as described previously [66,67]. The column effluent was introduced into an Orbitrap Fusion mass spectrometer (MS; Thermo Scientific, Waltham, MA) operated at an orbitrap resolution of 120k for MS1 and with data-dependent MS2 in a linear ion trap. Ion spray ionization was used in the negative mode throughout the entire assay run. Data was converted to mzML format and was analyzed by XCMS v3.00 under R v3.4.2 (R Core Team, 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Available at: https://www.R-project.org/) [68]. Chromatographically obtained m/z peaks were classified based on their retention time and were matched against an in silico generated lipid database. Lipid signals were corrected for 13C isotope contributions. An exemplary base peak chromatogram with identified phospholipids of one sample is shown as supplementary figure (S1 Fig).

Statistical analysis

In order to allow for a large number of measured phospholipids and to maintain practicality of the statistical analysis, yet allowing for the detection of significant differences, a data reduction was performed first by means of a principal component analysis (PCA), which was performed with the R package 'PCAMethods' (version 1.70.0) using the nonlinear iterative partial least squares (nipals) algorithm with pareto scaling [69]. Principal component analysis was performed on the complete data set, including all identified phospholipid species of both whole blood and plasma samples. These principal components (PrComp) were used as new variables for subsequent analyses. In the full dataset, the majority of phospholipidic variance could be explained by the type of specimen used. Thus, further statistical analysis of the obtained PrComps was performed separately for whole blood and plasma. In order to evaluate the effect of several different variables (including the aforementioned treatment of dogs with FRD or IBD, disease category, interaction of treatment and disease category, age, breed, weight, BCS, and sex) on the variance of the phospholipid profile, the R package 'LME4' (version 1.1-17) was used to compare the full linear model with mixed effects to the same model excluding specific factors or interactions. An analysis of variance (ANOVA) was then performed to assess the significance of the effect of a given factor. P values < 0.05 were considered statistically significant. The model used for comparisons was chosen according to the lowest Akaike information criterion (AIC) score, and both fixed (treatment, disease category, age, BCS, and weight, including the interaction between treatment and disease category) and random factors (breed, sex, and disease category nested within dog subject) were included in the model. Furthermore, all individual phospholipids were analyzed using the same linear mixed model as for the principal component analysis. The p-values obtained by this method were adjusted for false discovery rate using the Benjamini-Hochberg procedure. In addition, random forest analysis was performed using the web-based program MetaboAnalyst 4.0 to identify phospholipids that possibly contribute to the differentiation between disease category and also between treatment statuses.

Results

Animals

Thirty-two dogs were enrolled in the study. Sixteen of the dogs were categorized as FRD, as their clinical signs improved to the extent of being clinically insignificant (CIBDAI score 0-3) after dietary modification. The remaining 16 dogs required additional immunosuppressant treatment based on which these dogs were classified as having IBD. Tables 1 and S1 summarize the characteristics of the dogs enrolled in the study.

Table 1. Basic characteristics of the dogs included in the study (n = 32).

Disease	Breed	Age	Sex	Weight	BCS	CIBDAI
IBD, PLE	Papillon	3 y	mn	3.0 kg	3/9	14
IBD, PLE	Rottweiler	10 y	fs	31.6 kg	6/9	9
IBD	Golden Retriever	6 y 10 mo	mn	36.5 kg	6/9	7
IBD	Rottweiler	7 y 7 Mo	f	60.0 kg	8/9	6
IBD, PLE	Beauceron	4 y	fs	28.9 kg	n/a	14
IBD, PLE	Bernese Mountain Dog	5 y	fs	35.5 kg	n/a	12
IBD	American Cocker Spaniel	3 y 7 mo	mn	10.8 kg	5/9	6
IBD, PLE	Yorkshire Terrier/Shi Tzu mix	11 y	fs	4.0 kg	4/9	11
IBD	Mixed breed medium size	12 y 10 mo	mn	27.4 kg	5/9	3
IBD	Cavalier King Charles Spaniel	4 y 6 mo	m	8.6 kg	5/9	4
IBD, PLE	Pug	3 y 6 mo	m	11.2 kg	6/9	8
IBD, PLE	Mixed breed medium size	5 y 11 mo	mn	11.5 kg	3/9	n/a
IBD	Malinois	2 y 8 mo	mn	32.6 kg	4/9	15
IBD, PLE	Labrador mix	3 y	mn	23.2 kg	3/9	5
IBD, PLE	Pug	6 y 10 mo	f	7.2 kg	5/9	4
IBD	Mixed breed medium size	2 y 11 mo	fs	20.3 kg	5/9	4
FRD	Mixed breed medium size	3 y	mn	30.0 kg	7/9	9
FRD	Dachshund	3 y	fs	7.0 kg	4/9	5
FRD	Yorkshire Terrier	8 y 6 mo	fs	2.9 kg	6/9	6
FRD	French Bulldog	1 y 4 mo	m	14.6 kg	5/9	8
FRD	Weimaraner	2 y	fs	23.0 kg	4/9	4
FRD	Tervuren-Irish Wolfshound mix	9 mo	f	25.5 kg	4/9	5
FRD	Samoyed-Border Collie-Swiss Mountain Dog mix	5 y 10 mo	mn	23.0 kg	4/9	7
FRD	Cairn Terrier	3 y	m	9.4 kg	n/a	4
FRD	Golden Retriever	1 y 2 mo	f	20.1 kg	3/9	11
FRD	West Highland White Terrier	1 y	f	6.4 kg	6/9	4
FRD	Labrador Retriever	2 y	m	46.0 kg	6/9	9
FRD	Berger Blanc Suisse	2 y	fs	32.0 kg	n/a	8
FRD	Labrador	11 y 2 mo	mn	32.5 kg	6/9	4
FRD	Mixed breed large size	6 y 2 mo	fs	49.0 kg	7/9	1
FRD	Newfoundland	6 y 9 mo	m	44.2 kg	4/9	4
FRD	German Shepherd Dog	1 y 1 mo	m	34.3 kg	5/9	n/a

The canine inflammatory bowel disease activity index (CIBDAI) refers to the clinical activity score at the first visit.
The body condition score (BCS) refers to the body condition score of the first visit.
y, year; mo, months; f, female; m, male; n, neutered; s, spayed; n/a, not available

Nine dogs with IBD (56%) showed hypoalbuminemia and were diagnosed with protein-losing enteropathy as a result of severe lymphoplasmacytic inflammation due to idiopathic IBD. Three of these dogs significantly improved with prednisolone (1 mg/kg p.o. BID) monotherapy, while one dog required treatment with prednisolone and cyclosporine, and another dog required prednisolone and cyclosporine with prednisolone being tapered off and

replaced by budesonide later on. Four of the dogs with PLE (44%) were eventually euthanized due to clinical deterioration, with three dogs having received a combination of prednisolone and cyclosporine and one dog having received budesonide and cyclosporine. All other dogs with IBD and normoalbuminemia responded to prednisolone (starting dose 1 mg/kg p.o. BID) which could either be gradually reduced to only a small dose (e.g., 0.25 mg/kg p.o. EOD) or dogs could be taken off of prednisolone completely during the course of treatment. One dog, however, developed severe side effects of prednisolone and was switched to budesonide, which was better tolerated. A total of 24 dogs were included in the within-group evaluation of the effect of treatment on the phospholipid profile. In addition to the four dogs with PLE that were euthanized, one dog with FRD was censored from further analysis due to the owners declining a second endoscopy under general anesthesia. Another dog with FRD had to be excluded because of its refusal to eat the study diet, and two additional dogs with IBD were excluded from the study due to one owner declining further participation in the study and the other owner not following the study protocol.

Phospholipid profiles

Principal component analysis revealed a clear differentiation between the phospholipid profiles of whole blood and plasma regardless of the disease category (Fig 1), thus subsequent analyses were performed separately for whole blood and plasma. Furthermore, as PrComp1, PrComp2 and PrComp3 represented the principal components comprising the majority of the original phospholipid variance, these three PrComps were used for further statistical analysis.

Phospholipid analysis of whole blood did not identify any factors that significantly affected the variance of the phospholipid composition on PrComp1 (Table 2). Despite not reaching significance, breed seemed to have the biggest effect on the variance. However, on PrComp2 the phospholipid profile changed significantly depending on treatment status, with an increase in lysolipids (especially lysophosphatidylcholine [lysoPC] and lysophosphatidylinositols [lysoPI]) after initiation of treatment (Fig 2). Interestingly, after initiation of treatment the originally most abundant phosphatidylcholine (PC) species PC 38:4 (of which the most common molecular species is PC 18:0/20:4) was mainly converted into lysoPC 18:0 and arachidonic acid (i.e., 20:4 fatty acid) (S2 Fig), with a significant change in the ratio of PC 38:4 to lysoPC 18:0 ($p < 0.0024$, S4 Table). Furthermore, the effect of treatment depended on the disease category, and this effect was largest for dogs diagnosed with PLE due to IBD. Disease category and BCS also showed a significant effect on the phospholipid composition of whole blood in PrComp2 (Fig 3). In contrast, in PrComp3 none of the factors evaluated had a significant effect on the phospholipid profile (Table 2).

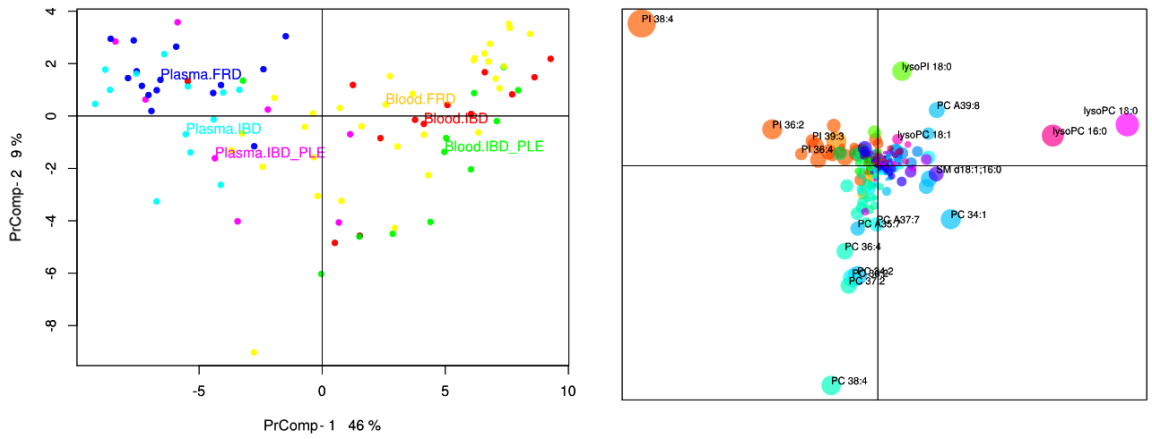

Fig 1. Principal component analysis (PCA) of the phospholipid profile in plasma and whole blood samples. In the score plot (left panel) each dot represents one analyzed sample and similar phospholipid profiles cluster together. A clear separation between plasma and blood samples is observed. In both types of specimen, the three disease categories are similarly positioned to each other, indicating a similar shift in the phospholipid profile in both sample types. Samples are color-coded by sample type and disease classification. The principal component loadings plot (right panel) visualizes the contribution of each phospholipid species to the total variance in the phospholipid profile, to which phospholipid species with the largest distance from the origin contributed the most. Each dot represents a different phospholipid species and the same color is used for the same phospholipid class. Dot sizes are proportional to the MS signal intensity of the phospholipid species. PrComp, principal component; PC, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol. https://doi.org/10.1371/journal.pone.0215435.g001

The analysis of phospholipids in plasma also revealed significant associations of specific variables and the composition of the phospholipid profile (Table 2). On PrComp1, both treatment and the interaction between treatment and disease category had a significant effect. Similar to whole blood, the most significant effects were detected on PrComp2 in plasma. In addition to treatment and the interaction of treatment with disease classification, age and weight also had an effect on the phospholipid composition (Fig 4). Treatment, disease category, the interaction between treatment and disease category, and body weight were all found to be significant predictors on PrComp3. Sex did not have a significant impact on the phospholipid profile in plasma nor in whole blood samples.

Table 2. Summary of ANOVA *p*-values of all evaluated parameters.

	***P*-value**		
	PrComp1	PrComp2	PrComp3
Whole blood			
Interaction treatment & disease category	0.3942	0.5353	0.0828
Treatment	0.7334	**0.0027**	0.2082
Disease category	0.8411	**0.0082**	0.1021
Weight	0.9088	0.1303	0.5920
Age	0.8647	0.3694	0.3668
BCS	0.6296	**0.0296**	0.4735
Breed	0.3097	**0.0081**	1.0000
Sex	1.0000	1.0000	1.0000
Plasma			
Interaction treatment & disease category	**0.0332**	**0.0472**	**0.0107**
Treatment	**0.0249**	**0.0238**	**0.0039**
Disease category	0.9593	0.1015	**0.0233**
Weight	0.6662	**0.0075**	**0.0155**
Age	0.1469	**0.0105**	0.5661
BCS	0.1467	0.4361	0.1654
Breed	0.9840	0.1100	1.0000
Sex	1.0000	1.0000	0.6163

PrComp, principal component; BCS, body condition score.
P values < 0.05 considered as significant, highlighted in bold print.

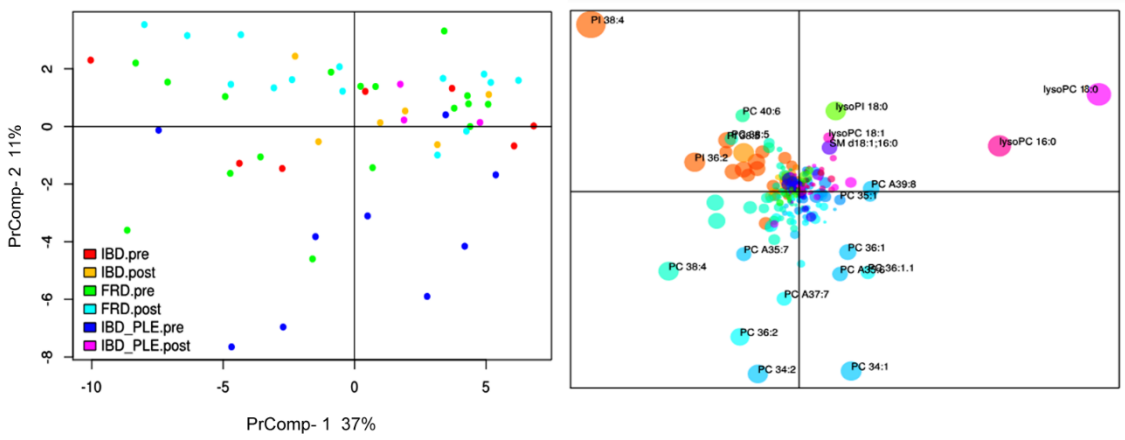

Fig 2. Principal component analysis (PCA) of the phospholipid profile in whole blood samples. In the score plot (left panel, for annotations see Fig 1) samples are color-coded by disease category and treatment status. The post-treatment samples are clustered in the upper right quadrant. In the corresponding loadings plot (right panel) lysolipid species are the predominant phospholipids in the upper right quadrant, indicating that the level of lysolipids increases after treatment. PrComp, principal component; PC, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol.
https://doi.org/10.1371/journal.pone.0215435.g002

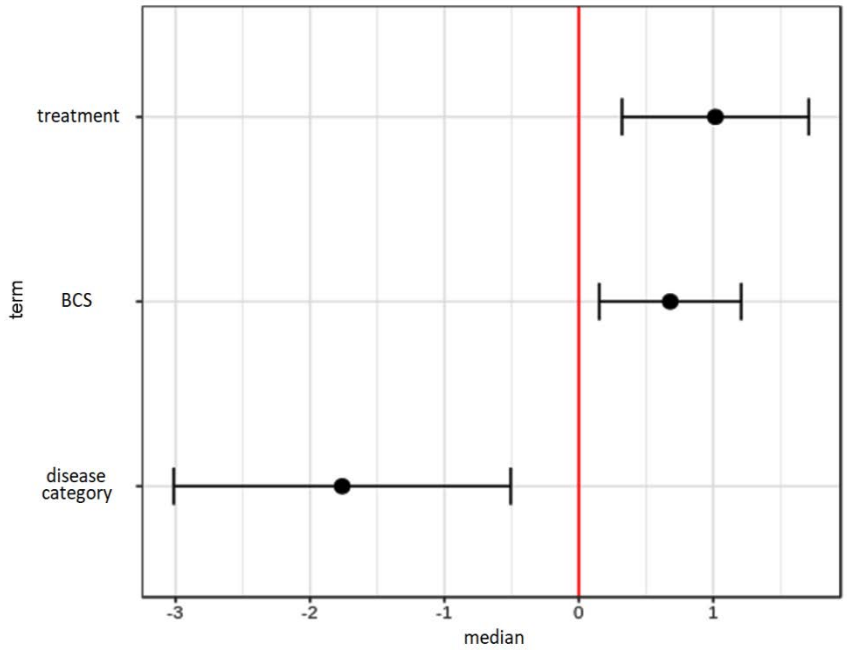

Fig 3. Effect of selected variables on variance of the phospholipid profile on PC2 in whole blood. Fixed effects are plotted with 95% confidence intervals. Treatment, body condition score (BCS), and disease category do not overlap with 0, thus representing significant factors on the variance of the phospholipid composition (ANOVA $p < 0.05$). https://doi.org/10.1371/journal.pone.0215435.g003

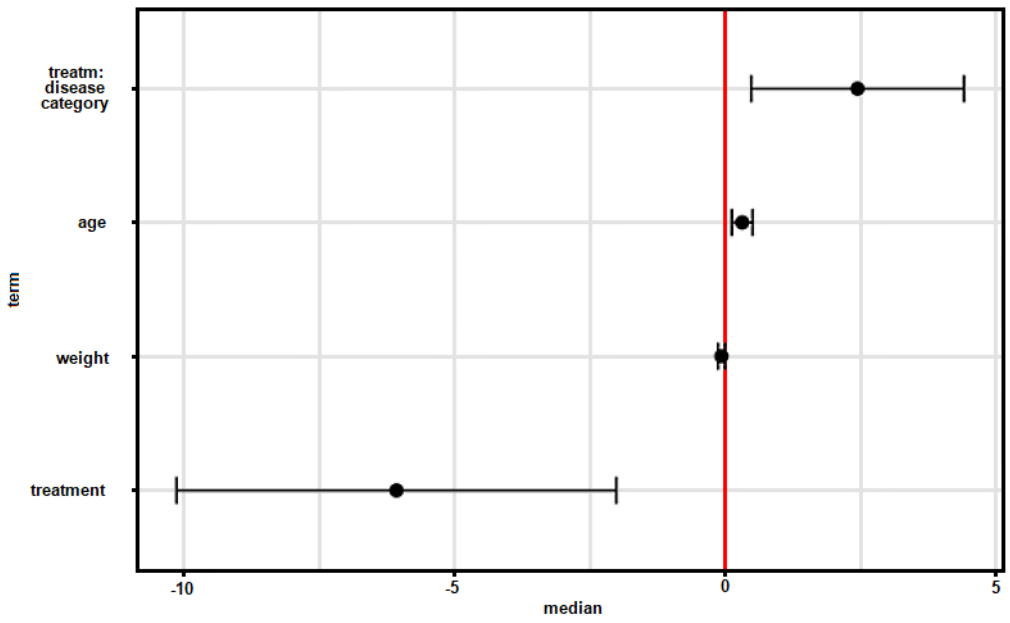

Fig 4. Effect of selected variables on variance of the phospholipid profile on PC2 in plasma. Fixed effects are plotted with 95% confidence intervals. The interaction between treatment and disease category, age, body weight, and treatment do not overlap with 0 and thus significantly affect the variance of the phospholipid composition (ANOVA $p < 0.05$). treatm:disease category, interaction of treatment and disease category.
https://doi.org/10.1371/journal.pone.0215435.g004

Random forest analysis revealed several phospholipids with the highest discriminatory power between disease categories and between treatment statuses within one disease category. Figs 5-7 depict the 15 most important phospholipids each that separate either between disease categories (Fig 5) or treatment statuses (Figs 6 and 7).

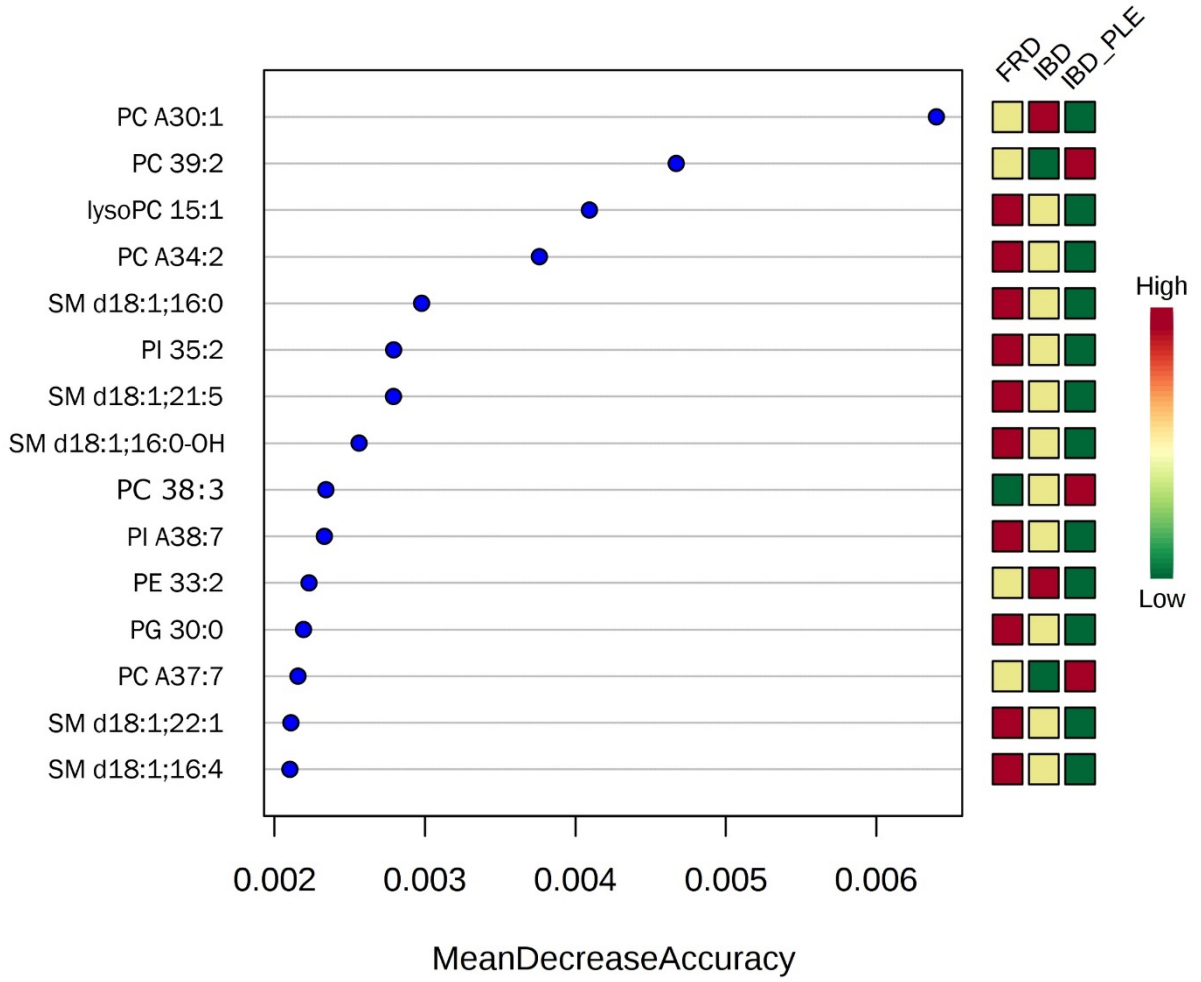

Fig 5. Random forest analysis of phospholipids and their association with disease category. Fifteen phospholipids with the highest discriminatory power between the disease categories are presented. The abundance of the phospholipids is color-coded, with red boxes representing a high abundance and green boxes representing a low abundance of a given phospholipid. PC, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol. 'A' indicates an ether species; the XX:y notation specifies the total number of carbon atoms in the radyl chains ('XX') followed by the number of unsaturation ('y'); for sphingolipids, a sphingosine (d18:1) backbone was assumed.
https://doi.org/10.1371/journal.pone.0215435.g005

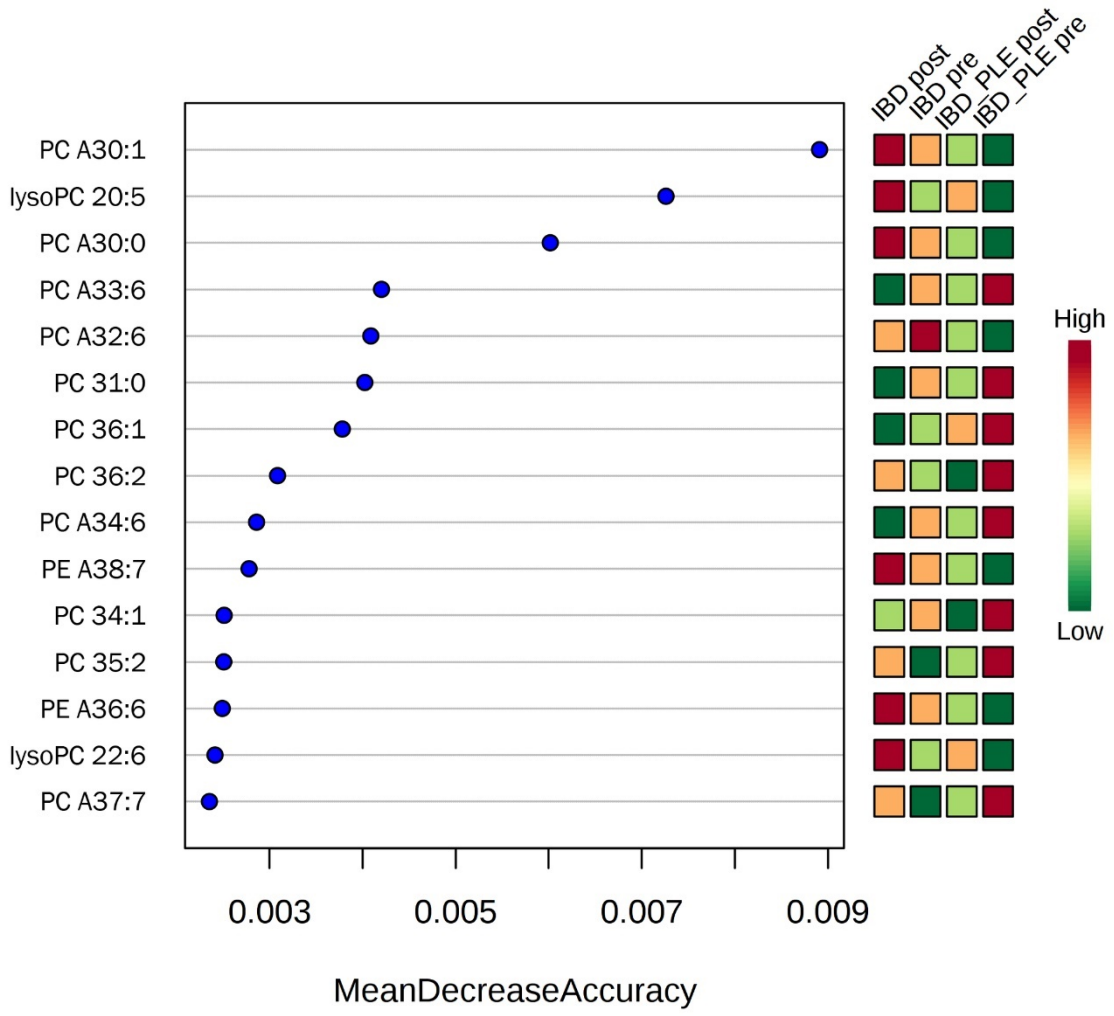

Fig 6. Random forest analysis of phospholipids and their association with treatment in IBD. The 15 top phospholipids based on their importance to discriminate between the treatment statuses of dogs with IBD are shown. Red boxes represent a high abundance, green boxes a low abundance of a given phospholipid. PC, phosphatidylcholine; PE, phosphatidylethanolamine; 'A' indicates an ether species; the XX:y notation specifies the total number of carbon atoms in the radyl chains ('XX') followed by the number of unsaturation ('y').
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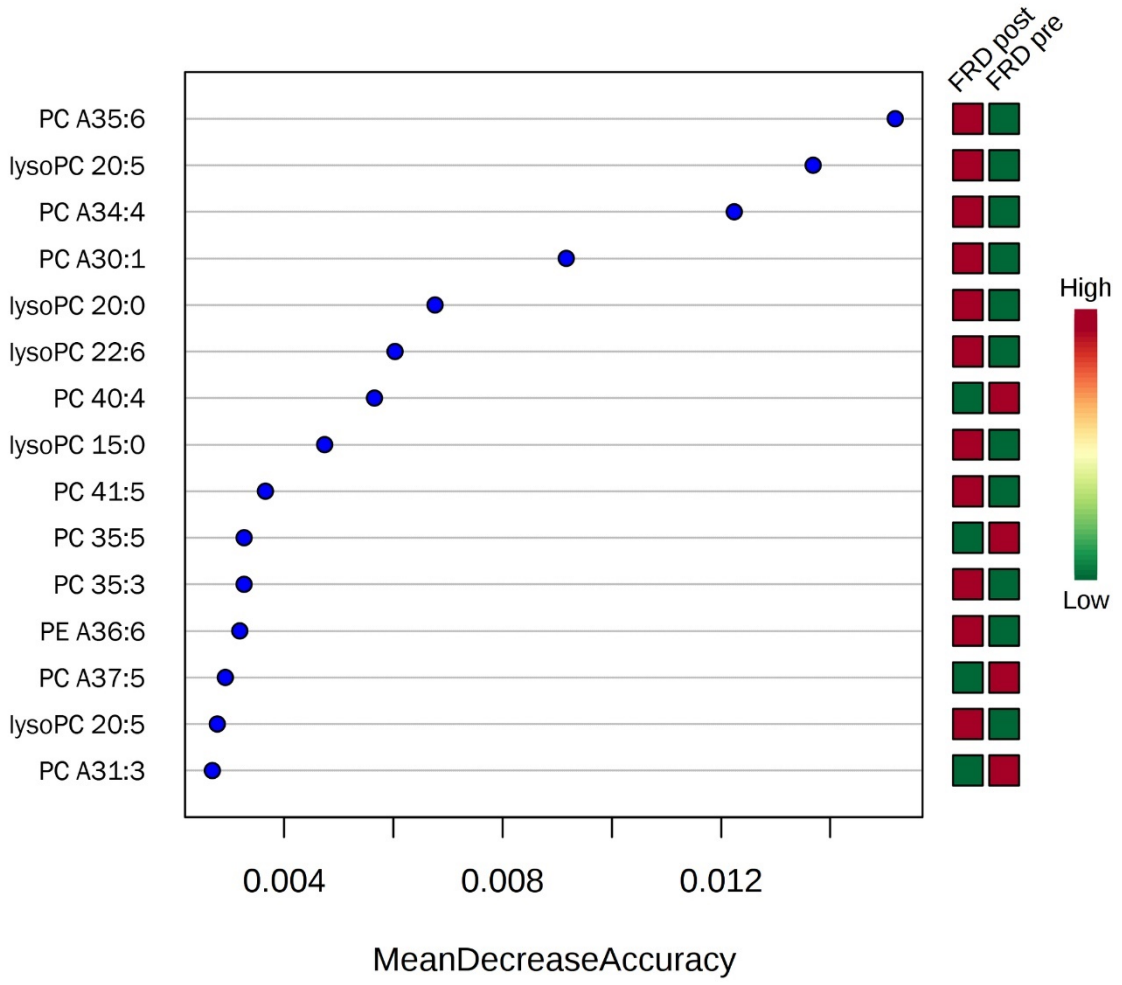

Fig 7. Random forest analysis of phospholipids and their association with treatment in FRD. The 15 top phospholipids based on their importance to discriminate between the treatment statuses of dogs with FRD are shown. Red boxes represent a high abundance, green boxes a low abundance of a given phospholipid. PC, phosphatidylcholine; PE, phosphatidylethanolamine; 'A' indicates an ether species; the XX:y notation specifies the total number of carbon atoms in the radyl chains ('XX') followed by the number of unsaturation ('y').
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Discussion

This is the first study to investigate the systemic phospholipid profile in samples from dogs diagnosed with IBD or FRD and to evaluate changes in the phospholipid profile following the initiation of treatment in these dogs. Significant differences were observed in the phospholipid profiles in dogs with CIE, especially between the two different types of specimen used (whole blood vs. plasma), disease category (IBD vs. FRD), and treatment status. An explanation for the distinct phospholipid profiles in whole blood and plasma samples as discovered by PCA could be the difference in the number of cells within these sample types. As lipids, including phospholipids, are essential components of cellular membranes [70,71], it appears reasonable to expect a difference in the phospholipid

composition between whole blood (which contains erythrocytes, leukocytes, and platelets) and plasma (which is essentially devoid of these cells but contains lipoproteins). An influence of different packed cell volumes or numbers of leukocytes of the patients on the findings of this study can currently not be fully excluded. In the current study, slightly more significant effects were found in plasma samples than in whole blood samples. This finding, in addition to a generally better storage stability of plasma [72] as well as less disturbance of the interpretation due to a lower number of unrelated lipids present in the cell membranes, leaves – in the authors' opinion – plasma the preferable sample type for future studies on the phospholipid profile.

Overall, treatment and disease category were the most significant variables affecting the phospholipid profiles. A significant shift of PC species was detected from PC 38:4 (18:0/20:4) before treatment to the corresponding lysolipid PC 18:0 after treatment. The loss of arachidonic acid (20:4) from the sn-2 position after initiation of treatment might be explained by an activation of phospholipase A2 and thus the liberation of arachidonic acid as a precursor for the synthesis of pro-inflammatory (e.g., prostaglandins and leukotrienes) or anti-inflammatory mediators (e.g., lipoxins and resolvins) [73-75]. Clinical improvement of most of the dogs after treatment would also render a shift towards anti-inflammatory mediators a likely explanation. Furthermore, therapeutic intervention in this study involved an elimination diet based on fish with additional enrichment of PUFAs. PUFAs have been shown to exert several anti-inflammatory actions [47,48,51,76]. Amongst others they decrease the production of arachidonic acid-derived pro-inflammatory eicosanoids (e.g., prostaglandin E2, 4-series leukotrienes) in favor of anti-inflammatory eicosanoids (e.g., prostaglandin E3, 5-series leukotrienes) and they generate further anti-inflammatory mediators such as resolvins and protectins [47,48,75,77]. Thus, the shift to lysoPC 18:0 after treatment might represent an increase in anti-inflammatory mediators, which may be associated with the amelioration of clinical signs observed in the dogs after treatment.

The significant effect of disease category on the phospholipid profile found in this study is interesting and might be due to the severity of the disease. This theory is supported by finding that the significant effect of treatment on the phospholipid profile in whole blood (see above) was linked to disease category, being largest for dogs diagnosed with IBD with PLE. Clinical severity usually increases from FRD to IBD to IBD with PLE [3,5]. Thus, the loss of 20:4 from PC appears to be largest in dogs with the most severe clinical signs. It also appears to be reasonable to assume that the amount of anti-inflammatory mediators required to counteract inflammation is higher with more severe disease. In the current study, disease severity and also clinical improvement were judged using the CIBDAI score. The canine chronic enteropathy clinical activity index (CCECAI), which is an extended scoring index that

included the additional criteria hypoalbuminemia, assessment of ascites, peripheral edema and pruritus, was introduced during the current study in 2007 [5], and an attempt was made to use this index for the present investigation. However, as a proper retrospective assessment of the dogs enrolled in this study prior to the CCECAI score's publication was not possible, and in order to obtain comparable results for all dogs, the CCECAI score was not further assessed in this study.

Age has been shown to affect the lipid profile [78,79]. In this study, dogs with IBD are significantly older than dogs with FRD. Hence, this difference in age could also have contributed to the significant effect of disease category on the phospholipid profile. However, dogs diagnosed with IBD have generally been reported to be older at the time of diagnosis than dogs diagnosed with FRD [3,5] and so, these study dogs represent a typical group of patients in a realistic clinical setting. Although age-matched study groups would have been desirable, the authors still consider the comparison of their representative groups of patients justifiable.

Random forest analysis identified some phospholipids which contribute to the separation of disease category and treatment status. When evaluating the different disease categories, sphingomyelin species were shown to be differentially abundant in dogs with FRD. Sphingomyelin has been reported to promote apoptosis in intestinal epithelial cells and to increase inflammation in mice with induced colitis [80]. The relevant abundance of sphingomyelin species in dogs with FRD could thus result from the inflammatory process of dogs with CIE. Yet, this finding raises the question why sphingomyelin was found to be of importance only in the least severe disease category. The authors, unfortunately, do not have a plausible explanation for this finding at the moment. Also, phosphatidylcholine species were important to differentiate between treatment statuses both in dogs with IBD and FRD. Former studies have demonstrated phosphatidylcholine to possess anti-inflammatory properties and to be beneficial in humans with ulcerative colitis [61,81]. Moreover, patients with ulcerative colitis were reported to have significantly lower levels of phosphatidylcholine and lysophosphatidylcholine compared to healthy controls [82,83]. In the current study, lysophosphatidylcholine species were also significantly relevant to differentiate between treatment statuses in dogs with FRD, with lysophosphatidylcholine species being important after treatment. As all dogs with FRD improved clinically after treatment, this finding is in line with previous studies. However, overall, the true relevance of the phospholipids identified on random forest analysis in the pathogenesis of CIE still remains elusive.

In the current study, the BCS only appeared to influence the phospholipid profile to some degree in whole blood, whereas body weight (but not BCS) presented a significant predictor of the phospholipidic variance in plasma. Previous studies have examined associations

between obesity and variations in the lipid profile. While one study found no significant difference in total cholesterol concentrations between obese dogs and lean controls [84], other studies did reveal a clear discrepancy in both cholesterol concentration and lipoprotein profiles when comparing overweight to healthy dogs [79,85]. Mori et al. also suggested that changes in plasma lipoprotein concentrations are more significant in older dogs with obesity (> 8 years of age) and that – as already mentioned above – both age and gender independently affect lipoprotein concentrations. Those findings are also in accordance with other studies [78,86]. In addition, lipid metabolism has been reported to be potentially influenced by breed [78]. The low impact of BCS and body weight on the phospholipid profile observed in the current study might be explained by the fact that the majority of dogs were assigned a BCS between 4/9 and 6/9 at their initial visit, representing a similar and close to ideal BCS in most dogs. Furthermore, as only three dogs were assigned a BCS ≥ 7/9, the influence of obesity could not be evaluated in this study cohort. Also, more advanced diagnostics to determine body fat (e.g., measured by dual-energy x-ray absorptiometry) were not performed in this study. Contrary to previous studies, sex had no effect on the lipid profile in the present study. Neither did the effect of breed reach significance, even though this was the most crucial effect on variance on PrComp1 in whole blood samples. The difference between our findings and those of others might be explained by the focus on different types of lipids. Whereas the current study examined phospholipids, former studies have mostly investigated concentrations of cholesterol and lipoproteins.

The lack of a control group presents a limitation of this study. However, previous studies have already evaluated the lipid profile in healthy dogs [84,87-91] and the main objective of the current study was to describe and compare the phospholipid profile of dogs with IBD or FRD before and after treatment. Nevertheless, a control group of dogs with CIE receiving the same treatment but short of PUFAs would have allowed for assessing the sole effect of supplemental PUFAs on the phospholipid profile and the dogs' clinical response. The stability of fatty acids during storage poses an additional limitation, as recent studies have reported fatty acids to be affected by degradation during long-term storage and have revealed storage at -80 °C to be the most stabilizing [72,92-94]. In this study, all samples had been stored frozen at -20 °C for approximately 10 years without discontinuity of the cooling chain. To the authors' knowledge, the exact mechanisms and kinetics of phospholipid degradation over a period of ten years have not been reported, but a similar risk of degradation is most likely for the current samples because all samples were subjected to the exact same storage conditions. An increase in lysolipids due to degradation cannot be excluded in this study, but the effect of storage on the phospholipid profile represents a methodic error, leaving – in the authors' opinion – the findings of this study (e.g., significant variance of the phospholipid profile depending on disease category) still reliable. Another shortcoming is the small study

cohort of our investigation. A larger number of dogs might have revealed a more distinct phospholipid signature in each disease group and with response to treatment. However, the number of dogs in this study was clearly affected by both the ethical aspect of performing a repeat endoscopy in dogs that had clinically improved as well as the willingness of the owners to consent to repeat examinations.

In addition, a confounding effect of the different time period between pre- and post-treatment evaluation in the two disease groups cannot be excluded in this study. However, as the treatment with immunosuppressant medication in dogs with IBD was started 14 days after the dietary modification and based on the absence of clinical improvement on the diet alone, those dogs had to be allowed more time to respond to treatment. Clinical signs in dogs with FRD typically improve faster on appropriate treatment, while dogs needing immunosuppressant treatment can take longer to respond. Thus, the different time scales were necessary to ensure correct classification of the disease status and were also chosen according to previous studies [5,11,62].

The duration of the elimination diet trial was set to 14 days. Potentially, few dogs with FRD could have not yet responded within that time period. However, this duration was chosen for the dietary trial according to current consensus that dogs with FRD typically show significant clinical improvement within 14 days of a strict dietary trial [3,5,95,96]. In addition, this time frame assisted with proper owner compliance. Similarly, few dogs with IBD may have improved on the diet at first and thus, could have been incorrectly classified as FRD. However, despite dietary modification being important in the management of canine IBD, it is the authors' experience that dogs with IBD respond only to a certain extent to an elimination diet alone. Thus, it appears to be unlikely that dogs with IBD showed either significant improvement or clinical remission on the elimination diet resulting in incorrect classification.

The histopathologic score was assigned in this study according to a publication by Jergens et al. [65]. The World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization Group released a new guideline for histopathological evaluation of gastrointestinal tissues in 2008 [97], which was revised and released as an ACVIM consensus statement in 2010 [10]. Similar to the extended clinical scoring system (CCECAI [5]), the 2008 WSAVA gastrointestinal histopathology guideline was not adopted during the course of the current study in order to apply the same histopathologic criteria to all dogs included.

Glucocorticoids, regardless of endogenous or exogenous excess, have been recognized to cause hyperlipidemia in dogs, mainly reflected in a mild hypercholesterolemia and hypertriglyceridemia and increased very low density lipoproteins [38,84,86,98,99]. Similarly, cyclosporine has been reported to cause hyperlipidemia characterized by

hypercholesterolemia and increased serum concentrations of low density lipoprotein cholesterol [100-102]. Furthermore, glucocorticoids are known to modulate arachidonic acid metabolism and alter membrane phospholipids [103,104]. Hence, an effect of immunosuppressant medication on the phospholipid profile cannot be excluded. As immunosuppressants are crucial in the treatment of canine IBD, this influence on the results could not be avoided. Comparison of the findings in this study to the plasma phospholipid composition in a healthy cohort receiving the study diet together with an immunosuppressant could have helped distinguishing the effects of the disease from those of the immunosuppressant on the plasma phospholipid profile. However, the use of immunosuppressant medications in healthy pet dogs was considered unethical by the authors and would have been also very difficult to get approved by the local ethics committee.

Finally, several studies have concluded that diet composition can considerably affect lipid metabolism and cholesterol concentrations [78,85]. A study by Jeusette et al. revealed that a nutritional modification to a high-protein low-energy diet had advantageous effects on plasma lipids in obese dogs [85]. Pasquini et al. described, amongst other findings, that serum cholesterol concentrations were the lowest in dogs fed a diet with a high content of fish. Also, diet composition has been shown to impact the phospholipid composition and characteristics of cell membranes (e.g., lipid rafts, membrane fluidity), rendering dietary phospholipids a potential therapeutic avenue [49,105,106]. Furthermore, an effect of the diet given prior to inclusion of a dog in the study on the pre-treatment systemic phospholipid profile remains a possibility. The dogs received a large variety of different diets including both dry and canned diets of various brands as well as home-made diets before entering the study. A standardization of the dogs' diets prior to enrollment in this study would have been extremely difficult if not impossible due to the variety of previous diets as well as the medical situation of the dogs presented, often showing severe clinical symptoms and the owner's understandably strong desire to obtain timely medical treatment – without first standardizing the patient's diet for a longer period of time. Hence, a meaningful comparison of the nutritional content of previous diets and the study diet would have been ideal, but unfortunately was not feasible given the medical conditions of the dogs in this study.

In summary, this is the first study to analyze the systemic phospholipid profile in dogs with IBD or FRD before and also after treatment. The severity of the disease and the effect of treatment most significantly determined the composition of the phospholipid profile, and a significant shift in the phospholipid species was observed after treatment (PC 38:4 to lysoPC 18:0). These findings suggest that the phospholipid profile is an informative tool and might have clinical utility for evaluating the response to treatment. Future studies with improved

sample storage conditions are warranted to verify the results of this study in a larger group of dogs, to further investigate the association of different lipids with the pathophysiology of canine CIE, and to evaluate their potential as a novel therapeutic approach to canine IBD and FRD.

References

1. Hall EJ, German AJ (2010) Diseases of the Small Intestine. In: & ESJ, E.C. F, editors. Textbook of Veterinary Internal Medicine. 7th ed. St. Louis, Missouri, USA: Saunders Elsevier. pp. 1560-1566.

2. Dandrieux JR (2016) Inflammatory bowel disease versus chronic enteropathy in dogs: are they one and the same? J Small Anim Pract 57: 589-599.

3. Allenspach K, Culverwell C, Chan D (2016) Long-term outcome in dogs with chronic enteropathies: 203 cases. Vet Rec 178: 368.

4. Erdmann C, Heilmann RM (2017) Diagnostic and therapeutic approach to chronic inflammatory enteropathies in dogs. Tierarztl Prax Ausg K Kleintiere Heimtiere 45: 317-327.

5. Allenspach K, Wieland B, Gröne A, Gaschen F (2007) Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. J Vet Intern Med 21: 700-708.

6. Mandigers PJ, Biourge V, van den Ingh TS, Ankringa N, German AJ (2010) A randomized, open-label, positively-controlled field trial of a hydrolyzed protein diet in dogs with chronic small bowel enteropathy. J Vet Intern Med 24: 1350-1357.

7. German AJ, Hall EJ, Day MJ (2003) Chronic intestinal inflammation and intestinal disease in dogs. J Vet Intern Med 17: 8-20.

8. Westermarck E, Skrzypczak T, Harmoinen J, Steiner JM, Ruaux CG, et al. (2005) Tylosin-responsive chronic diarrhea in dogs. J Vet Intern Med 19: 177-186.

9. Kilpinen S, Spillmann T, Syrja P, Skrzypczak T, Louhelainen M, et al. (2011) Effect of tylosin on dogs with suspected tylosin-responsive diarrhea: a placebo-controlled, randomized, double-blinded, prospective clinical trial. Acta Vet Scand 53: 26.

10. Washabau RJ, Day MJ, Willard MD, Hall EJ, Jergens AE, et al. (2010) Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. J Vet Intern Med 24: 10-26.

11. Burgener IA, König A, Allenspach K, Sauter SN, Boisclair J, et al. (2008) Upregulation of toll-like receptors in chronic enteropathies in dogs. J Vet Intern Med 22: 553-560.

12. Kathrani A, House A, Catchpole B, Murphy A, German A, et al. (2010) Polymorphisms in the TLR4 and TLR5 gene are significantly associated with inflammatory bowel disease in German shepherd dogs. PLoS ONE 5: e15740.

13. Kathrani A, House A, Catchpole B, Murphy A, Werling D, et al. (2011) Breed-independent toll-like receptor 5 polymorphisms show association with canine inflammatory bowel disease. Tissue Antigens 78: 94-101.

14. Kathrani A, Lee H, White C, Catchpole B, Murphy A, et al. (2014) Association between nucleotide oligomerisation domain two (Nod2) gene polymorphisms and canine inflammatory bowel disease. Vet Immunol Immunopathol 161: 32-41.

15. Allenspach K, House A, Smith K, McNeill FM, Hendricks A, et al. (2010) Evaluation of mucosal bacteria and histopathology, clinical disease activity and expression of Toll-like receptors in German shepherd dogs with chronic enteropathies. Vet Microbiol 146: 326-335.

16. Simpson KW, Jergens AE (2011) Pitfalls and progress in the diagnosis and management of canine inflammatory bowel disease. Vet Clin North Am Small Anim Pract 41: 381-398.

17. Heilmann RM, Allenspach K (2017) Pattern-recognition receptors: signaling pathways and dysregulation in canine chronic enteropathies-brief review. J Vet Diagn Invest: 1040638717728545.

18. Minamoto Y, Otoni CC, Steelman SM, Buyukleblebici O, Steiner JM, et al. (2015) Alteration of the fecal microbiota and serum metabolite profiles in dogs with idiopathic inflammatory bowel disease. Gut microbes 6: 33-47.

19. Cassmann E, White R, Atherly T, Wang C, Sun Y, et al. (2016) Alterations of the Ileal and Colonic Mucosal Microbiota in Canine Chronic Enteropathies. PLoS ONE 11: e0147321.

20. Suchodolski JS, Dowd SE, Wilke V, Steiner JM, Jergens AE (2012) 16S rRNA gene pyrosequencing reveals bacterial dysbiosis in the duodenum of dogs with idiopathic inflammatory bowel disease. PLoS ONE 7: e39333.

21. Schmitz S, Suchodolski J (2016) Understanding the canine intestinal microbiota and its modification by pro-, pre- and synbiotics - what is the evidence? Vet Med Sci 2: 71-94.

22. Schmitz S, Glanemann B, Garden OA, Brooks H, Chang YM, et al. (2015a) A prospective, randomized, blinded, placebo-controlled pilot study on the effect of Enterococcus faecium on clinical activity and intestinal gene expression in canine food-responsive chronic enteropathy. J Vet Intern Med 29: 533-543.

23. Rossi G, Pengo G, Caldin M, Palumbo Piccionello A, Steiner JM, et al. (2014) Comparison of microbiological, histological, and immunomodulatory parameters in response to treatment with either combination therapy with prednisone and metronidazole or probiotic VSL#3 strains in dogs with idiopathic inflammatory bowel disease. PLoS ONE 9: e94699.

24. White R, Atherly T, Guard B, Rossi G, Wang C, et al. (2017) Randomized, controlled trial evaluating the effect of multi-strain probiotic on the mucosal microbiota in canine idiopathic inflammatory bowel disease. Gut microbes 8: 451-466.

25. Rossi G, Cerquetella M, Scarpona S, Pengo G, Fettucciari K, et al. (2018) Effects of probiotic bacteria on mucosal polyamines levels in dogs with IBD and colonic polyps: a preliminary study. Benef Microbes 9: 247-255.

26. Schmitz S, Werling D, Allenspach K (2015b) Effects of ex-vivo and in-vivo treatment with probiotics on the inflammasome in dogs with chronic enteropathy. PLoS ONE 10: e0120779.

27. Redfern A, Suchodolski J, Jergens A (2017) Role of the gastrointestinal microbiota in small animal health and disease. Vet Rec 181: 370.

28. Murphy T, Chaitman J, Han E (2014) Use of fecal transplant in eight dogs with refractory Clostridium perfringens associated diarrhea. J Vet Intern Med 28: 976-1134.

29. Chaitman J, Jergens AE, Gaschen F, Garcia-Mazcorro JF, Marks SL, et al. (2016) Commentary on key aspects of fecal microbiota transplantation in small animal practice. Vet Med (Auckl) 7: 71-74.

30. Lam SM, Shui G (2013) Lipidomics as a principal tool for advancing biomedical research. J Genet Genomics 40: 375-390.

31. Kjellqvist S, Klose C, Surma MA, Hindy G, Mollet IG, et al. (2016) Identification of Shared and Unique Serum Lipid Profiles in Diabetes Mellitus and Myocardial Infarction. J Am Heart Assoc 5: e004503.

32. He X, Huang Y, Li B, Gong CX, Schuchman EH (2010) Deregulation of sphingolipid metabolism in Alzheimer's disease. Neurobiol Aging 31: 398-408.

33. Liu Q, Zhang J (2014) Lipid metabolism in Alzheimer's disease. Neurosci Bull 30: 331-345.

34. Fiorenza AM, Branchi A, Sommariva D (2000) Serum lipoprotein profile in patients with cancer. A comparison with non-cancer subjects. Int J Clin Lab Res 30: 141-145.

35. Fan F, Mundra PA, Fang L, Galvin A, Moore XL, et al. (2015) Lipidomic profiling in inflammatory bowel disease: comparison between ulcerative colitis and Crohn's disease. Inflamm Bowel Dis 21: 1511-1518.

36. Agouridis AP, Elisaf M, Milionis HJ (2011) An overview of lipid abnormalities in patients with inflammatory bowel disease. Ann Gastroenterol 24: 181-187.

37. Romanato G, Scarpa M, Angriman I, Faggian D, Ruffolo C, et al. (2009) Plasma lipids and inflammation in active inflammatory bowel diseases. Aliment Pharmacol Ther 29: 298-307.

38. Xenoulis PG, Steiner JM (2015) Canine hyperlipidaemia. J Small Anim Pract 56: 595-605.

39. Xenoulis PG, Suchodolski JS, Levinski MD, Steiner JM (2007) Investigation of hypertriglyceridemia in healthy Miniature Schnauzers. J Vet Intern Med 21: 1224-1230.

40. Seage EC, Drobatz KJ, Hess RS (2018) Spectrophotometry and ultracentrifugation for measurement of plasma lipids in dogs with diabetes mellitus. J Vet Intern Med 32: 93-98.

41. Yilmaz Z, Senturk S (2007) Characterisation of lipid profiles in dogs with parvoviral enteritis. J Small Anim Pract 48: 643-650.

42. Dill AL, Ifa DR, Manicke NE, Costa AB, Ramos-Vara JA, et al. (2009) Lipid profiles of canine invasive transitional cell carcinoma of the urinary bladder and adjacent normal tissue by desorption electrospray ionization imaging mass spectrometry. Anal Chem 81: 8758-8764.

43. Behling-Kelly E (2014) Serum lipoprotein changes in dogs with renal disease. J Vet Intern Med 28: 1692-1698.

44. Smith RE, Granick JL, Stauthammer CD, Polzin DJ, Heinrich DA, et al. (2017) Clinical consequences of hypertriglyceridemia-associated proteinuria in Miniature Schnauzers. J Vet Intern Med 31: 1740-1748.

45. Gültekin M, Pasa S, Ural K, Balikci C, Ekren Aşici G, et al. (2017) Oxidative status and lipid profile among dogs at different stages of visceral Leishmaniasis. Türkiye Parazitol Derg 41: 183-187.

46. Simopoulos AP (2002) Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr 21: 495-505.

47. Calder PC (2013) Omega-3 polyunsaturated fatty acids and inflammatory processes: nutrition or pharmacology? Br J Clin Pharmacol 75: 645-662.

48. Ungaro F, Rubbino F, Danese S, D'Alessio S (2017) Actors and factors in the resolution of intestinal inflammation: Lipid mediators as a new approach to therapy in inflammatory bowel diseases. Front Immunol 8: 1331.

49. Mueller RS, Fieseler KV, Fettman MJ, Zabel S, Rosychuk RA, et al. (2004) Effect of omega-3 fatty acids on canine atopic dermatitis. J Small Anim Pract 45: 293-297.

50. Bauer JE (2011) Therapeutic use of fish oils in companion animals. J Am Vet Med Assoc 239: 1441-1451.

51. Charpentier C, Chan R, Salameh E, Mbodji K, Ueno A, et al. (2018) Dietary n-3 PUFA may attenuate experimental colitis. Mediators Inflamm 2018: 8430614.

52. Ontsouka CE, Burgener IA, Mani O, Albrecht C (2010) Polyunsaturated fatty acid-enriched diets used for the treatment of canine chronic enteropathies decrease the abundance of selected genes of cholesterol homeostasis. Domest Anim Endocrinol 38: 32-37.

53. Ontsouka EC, Burgener IA, Luckschander-Zeller N, Blum JW, Albrecht C (2012) Fish-meal diet enriched with omega-3 PUFA and treatment of canine chronic enteropathies. Eur J Lipid Sci Technol 114: 412-422.

54. Küllenberg D, Taylor LA, Schneider M, Massing U (2012) Health effects of dietary phospholipids. Lipids in Health and Disease 11: 3-3.

55. Lordan R, Tsoupras A, Zabetakis I (2017) Phospholipids of animal and marine origin: structure, function, and anti-inflammatory properties. Molecules 22: 1964.

56. Tessaro FH, Ayala TS, Martins JO (2015) Lipid mediators are critical in resolving inflammation: a review of the emerging roles of eicosanoids in diabetes mellitus. Biomed Res Int 2015: 568408.

57. Gundermann KJ, Kuenker A, Kuntz E, Drozdzik M (2011) Activity of essential phospholipids (EPL) from soybean in liver diseases. Pharmacol Rep 63: 643-659.

58. Sahebkar A (2013) Fat lowers fat: purified phospholipids as emerging therapies for dyslipidemia. Biochim Biophys Acta 1831: 887-893.

59. Taylor LA, Pletschen L, Arends J, Unger C, Massing U (2010) Marine phospholipids—a promising new dietary approach to tumor-associated weight loss.

Support Care Cancer 18: 159.

60. Richter Y, Herzog Y, Lifshitz Y, Hayun R, Zchut S (2013) The effect of soybean-derived phosphatidylserine on cognitive performance in elderly with subjective memory complaints: a pilot study. Clin Interv Aging 8: 557-563.

61. Karner M, Kocjan A, Stein J, Schreiber S, von Boyen G, et al. (2014) First multicenter study of modified release phosphatidylcholine "LT-02" in ulcerative colitis: a randomized, placebo-controlled trial in mesalazine-refractory courses. Am J Gastroenterol 109: 1041-1051.

62. Dumusc SD, Ontsouka EC, Schnyder M, Hartnack S, Albrecht C, et al. (2014) Cyclooxygenase-2 and 5-lipoxygenase in dogs with chronic enteropathies. J Vet Intern Med 28: 1684-1691.

63. Laflamme D (1997) Development and validation of a body condition score system for dogs. Canine Pract 22: 10-15.

64. Jergens AE, Schreiner CA, Frank DE, Niyo Y, Ahrens FE, et al. (2003) A scoring index for disease activity in canine inflammatory bowel disease. J Vet Intern Med 17: 291-297.

65. Jergens A, Moore F, Haynes J, Miles K (1992) Idiopathic inflammatory bowel disease in dogs and cats: 84 cases (1987-1990). J Am Vet Med Assoc 201: 1603-1608.

66. Brouwers JF, Aalberts M, Jansen JW, van Niel G, Wauben MH, et al. (2013) Distinct lipid compositions of two types of human prostasomes. Proteomics 13: 1660-1666.

67. Jeucken A, Brouwers JF (2019) High-throughput screening of lipidomic adaptations in cultured cells. Biomolecules 9: 42.

68. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G (2006) XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal Chem 78: 779-787.

69. Stacklies W, Redestig H, Scholz M, Walther D, Selbig J (2007) pcaMethods--a bioconductor package providing PCA methods for incomplete data. Bioinformatics 23: 1164-1167.

70. De Gier J, Van Deenen LLM (1961) Some lipid characteristics of red cell membranes of various animal species. Biochim Biophys Acta 49: 286-296.

71. Schroit AJ, Zwaal RFA (1991) Transbilayer movement of phospholipids in red cell and platelet membranes. Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes 1071: 313-329.

72. Brenna JT, Plourde M, Stark KD, Jones PJ, Lin YH (2018) Best practices for the design, laboratory analysis, and reporting of trials involving fatty acids. Am J Clin Nutr 108: 211-227.

73. Kuehl F, Egan R (1980) Prostaglandins, arachidonic acid, and inflammation. Science 210: 978-984.

74. Serhan CN, Hamberg M, Samuelsson B (1984) Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human leukocytes. Proc Natl Acad Sci U S A 81: 5335-5339.

75. Bannenberg GL, Chiang N, Ariel A, Arita M, Tjonahen E, et al. (2005) Molecular circuits of resolution: formation and actions of resolvins and protectins. J Immunol 174: 4345-4355.

76. Shores DR, Binion DG, Freeman BA, Baker PR (2011) New insights into the role of fatty acids in the pathogenesis and resolution of inflammatory bowel disease. Inflamm Bowel Dis 17: 2192-2204.

77. Serhan CN, Clish CB, Brannon J, Colgan SP, Chiang N, et al. (2000) Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2–nonsteroidal antiinflammatory drugs and transcellular processing. J Exp Med 192: 1197-1204.

78. Pasquini A, Luchetti E, Cardini G (2008) Plasma lipoprotein concentrations in the dog: the effects of gender, age, breed and diet. J Anim Physiol Anim Nutr (Berl) 92: 718-722.

79. Mori N, Lee P, Kondo K, Kido T, Saito T, et al. (2011) Potential use of cholesterol lipoprotein profile to confirm obesity status in dogs. Vet Res Commun 35: 223-235.

80. Fischbeck A, Leucht K, Frey-Wagner I, Bentz S, Pesch T, et al. (2011) Sphingomyelin induces cathepsin D-mediated apoptosis in intestinal epithelial cells and increases inflammation in DSS colitis. Gut 60: 55-65.

81. Treede I, Braun A, Sparla R, Kuhnel M, Giese T, et al. (2007) Anti-inflammatory effects of phosphatidylcholine. J Biol Chem 282: 27155-27164.

82. Braun A, Treede I, Gotthardt D, Tietje A, Zahn A, et al. (2009) Alterations of phospholipid concentration and species composition of the intestinal mucus barrier in ulcerative colitis: a clue to pathogenesis. Inflamm Bowel Dis 15: 1705-1720.

83. Titz B, Gadaleta RM, Lo Sasso G, Elamin A, Ekroos K, et al. (2018) Proteomics and lipidomics in inflammatory bowel disease research: from mechanistic insights to biomarker identification. Int J Mol Sci 19: 2775.

84. Jericó MM, De Camargo Chiquito F, Kajihara K, Moreira MA, Gonzales R, et al. (2009) Chromatographic analysis of lipid fractions in healthy dogs and dogs with obesity or hyperadrenocorticism. J Vet Diagn Invest 21: 203-207.

85. Jeusette IC, Lhoest ET, Istasse LP, Diez MO (2005) Influence of obesity on plasma lipid and lipoprotein concentrations in dogs. Am J Vet Res 66: 81-86.

86. Barrie J, Watson TDG, Stear MJ, Nash AS (1993) Plasma cholesterol and lipoprotein concentrations in the dog: The effects of age, breed, gender and endocrine disease. J Small Anim Pract 34: 507-512.

87. Mahley RW, Weisgraber KH (1974) Canine lipoproteins and atherosclerosis: I. isolation and characterization of plasma lipoproteins from control dogs. Circ Res 35: 713-721.

88. Downs LG, Bolton CH, Crispin SM, Wills JM (1993) Plasma lipoprotein lipids in five different breeds of dogs. Res Vet Sci 54: 63-67.

89. Maldonado EN, Romero JR, Ochoa B, Aveldano MI (2001) Lipid and fatty acid composition of canine lipoproteins. Comp Biochem Physiol B Biochem Mol Biol 128: 719-729.

90. Xenoulis PG, Steiner JM (2010) Lipid metabolism and hyperlipidemia in dogs. Vet J 183: 12-21.

91. Xenoulis PG, Cammarata PJ, Walzem RL, Macfarlane RD, Suchodolski JS, et al. (2013) Novel lipoprotein density profiling in healthy dogs of various breeds, healthy Miniature Schnauzers, and Miniature Schnauzers with hyperlipidemia. BMC Vet Res 9: 47.

92. Metherel AH, Aristizabal Henao JJ, Stark KD (2013) EPA and DHA levels in whole blood decrease more rapidly when stored at -20 degrees C as compared with room temperature, 4 and -75 degrees C. Lipids 48: 1079-1091.

93. Metherel AH, Stark KD (2016) The stability of blood fatty acids during storage and potential mechanisms of degradation: A review. Prostaglandins Leukot Essent Fatty Acids 104: 33-43.

94. Matthan NR, Ip B, Resteghini N, Ausman LM, Lichtenstein AH (2010) Long-term fatty acid stability in human serum cholesteryl ester, triglyceride, and phospholipid fractions. J Lipid Res 51: 2826-2832.

95. Marks S, Laflamme DP, McAloose D (2002) Dietary trial using a commercial hypoallergenic diet containing hydrolyzed protein for dogs with inflammatory bowel disease. Vet Ther 3: 109-118.

96. Gaschen FP, Merchant SR (2011) Adverse food reactions in dogs and cats. Vet Clin North Am Small Anim Pract 41: 361-379.

97. Day MJ, Bilzer T, Mansell J, Wilcock B, Hall EJ, et al. (2008) Histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from the dog and cat: a report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group. J Comp Pathol 138 Suppl 1: S1-43.

98. Johnson MC (2005) Hyperlipidemia disorders in dogs. Compend Contin Educ Vet 27: 361-370.

99. Yavuz O, Turktas I, Cevik C (1996) The effect of high-dose inhaled budesonide on lipid profile in asthmatic patients. Gen Pharmacol 27: 89-90.

100. Reuben A (2001) Long-term management of the liver transplant patient: diabetes, hyperlipidemia, and obesity. Liver Transpl 7: S13-21.

101. Wasan KM, Brocks DR, Lee SD, Sachs-Barrable K, Thornton SJ (2008) Impact of lipoproteins on the biological activity and disposition of hydrophobic drugs: implications for drug discovery. Nat Rev Drug Discov 7: 84-99.

102. Steffan J, Parks C, Seewald W (2005) Clinical trial evaluating the efficacy and safety of cyclosporine in dogs with atopic dermatitis. J Am Vet Med Assoc 226: 1855-1863.

103. Goppelt-Struebe M, Wolter D, Resch K (1989) Glucocorticoids inhibit prostaglandin synthesis not only at the level of phospholipase A2 but also at the level of cyclo-oxygenase/PGE isomerase. Br J Pharmacol 98: 1287-1295.

104. Nelson DH (1980) Corticosteroid-induced changes in phospholipid membranes as mediators of their action. Endocr Rev 1: 180-199.

105. Goldberg RJ, Katz J (2007) A meta-analysis of the analgesic effects of omega-3 polyunsaturated fatty acid supplementation for inflammatory joint pain. Pain 129: 210-223.

106. Marion-Letellier R, Savoye G, Beck PL, Panaccione R, Ghosh S (2013) Polyunsaturated fatty acids in inflammatory bowel diseases: a reappraisal of effects and therapeutic approaches. Inflamm Bowel Dis 19: 650-661.

Supporting Information

S1 Fig. Base peak chromatogram of one of the samples. Base peak chromatogram of the separation by hydrophilic interaction liquid chromatography of phospholipids extracted from blood of a dog with FRD before treatment. Colored dots indicate retention time and m/z ratio of phospholipids detected by orbitrap ultrahigh resolution mass spectrometry.

S2 Fig. Effect of treatment on the individual phospholipids. The dashed red horizontal line is located at p = 0.05, with dots above the line having p-values < 0.05; p values < 0.05 considered as significant.

S1 Table. Characteristics of the dogs with IBD (n=16) or FRD (n=16) included in the study.

S2 Table. Raw peaklist of annotated phospholipids. Raw peaklist including the numbers of all 331 phospholipid signals in the samples analyzed. Phospholipids were annotated based on retention time and mass to charge (m/z) ratio. Phospholipids annotated with an '*' had a difference between theoretical and observed m/z of > 0.015 Da (but < 0.050) and should be considered 'tentatively identified'. Retention times and observed m/z values are included in the peaklist. Disease category: red = IBD; blue = IBD with PLE; green = FRD.

S3 Table. Sample identification and patient information.

S4 Table. P-values of effect of treatment, disease category and sample type on individual phospholipids.

S1 File. Nutritional composition of the study diet.

S2 File. Table of nutritional content (original in French).

S3 File. Results of external PUFA analysis by Swiss reference laboratory (original in French).

4 DISCUSSION

4.1 Objective of this Study

The aim of this study was, first, to compare the mucosal microbiota in the duodenum and colon between dogs diagnosed with IBD and dogs with FRD. Secondly, the duodenal and colonic mucosal microbiota in each dog was evaluated before and after the induction phase of treatment in order to evaluate the possibility of an effect of treatment on the intestinal microbial composition. The third objective of our study was to comparatively investigate the systemic phospholipid profile (i.e., the lipidome) of dogs with FRD and dogs with IBD, both before and after treatment including supplemental PUFAs.

Several studies have revealed alterations in the intestinal microbiota in dogs with IBD by examining fecal specimens (SUCHODOLSKI et al. 2012b, MINAMOTO et al. 2015, XU et al. 2016) or brush cytology samples (XENOULIS et al. 2008). To date, only few studies have used endoscopic biopsy samples to assess the mucosal microbial composition in dogs with IBD, whereby these studies were focused either on the duodenum (SUCHODOLSKI et al. 2010, SUCHODOLSKI et al. 2012a) or the ileum and colon (CASSMANN et al. 2016) only. To the author's knowledge, this study is the first to evaluate the intestinal microbiota of dogs with IBD examining both duodenal and colonic mucosal biopsies. Additionally, it is the first study to compare the intestinal microbiota of dogs with IBD and FRD, and also with consideration of the treatment status.

Furthermore, the lipid profile and potential alterations thereof have been the subject of a number of studies in healthy dogs (DOWNS et al. 1993, PASQUINI et al. 2008) and dogs with various diseases, such as hypertriglyceridemia (XENOULIS and STEINER 2015), endocrine disorders (BARRIE et al. 1993, SEAGE et al. 2018), obesity (JEUSETTE et al. 2005), cancer (DILL et al. 2009, TAMAI et al. 2014), parvovirus infection (YILMAZ and SENTURK 2007), renal disease (BEHLING-KELLY 2014), and infection with *Leishmania* (GÜLTEKIN et al. 2017). However, these previous studies investigated mainly the serum concentrations of cholesterol and lipoproteins. To date, there is no report about the circulating lipid profile of dogs with chronic inflammatory enteropathies (CIE). Thus, the current study is the first to evaluate the systemic phospholipid profile in dogs with IBD or FRD both prior to and after the induction phase of treatment.

Both materials and methods as well as the results of these studies have been released and also discussed in the two publications. This closing discussion aims to further elaborate on selected aspects of our findings and to summarize the results and conclusions of these studies.

4.2 Important Aspects of the Evaluation of the Intestinal Microbiota in Dogs with IBD and FRD

4.2.1 Further Discussion of the Results of the Microbiome Analysis

The global species richness was not significantly different between dogs diagnosed with IBD and dogs with FRD, neither in the mucosa of the duodenum nor in the colon. Also, within each disease group, significant differences in species richness were neither seen before nor after the induction phase of treatment. Furthermore, no significant differences in the overall microbial communities were identified based on the clinical disease severity – assessed by the CIBDAI score – prior to treatment. This is in accordance with a previous study, which reported no significant differences in the global composition of the intestinal microbiota between dogs with severe IBD and dogs with moderate IBD (SUCHODOLSKI et al. 2012a). These findings may be explained by the fact that both IBD and FRD represent inflammatory conditions with overlapping histological features (DAY et al. 2008) that can only be differentiated based on the response to treatment (ALLENSPACH et al. 2016, DANDRIEUX 2016). Thus, a comparable type and degree of histologic inflammation might affect the intestinal microbiome in a comparable manner. A lack of an association between the clinical disease activity score and histologic severity of disease (ALLENSPACH et al. 2007) would also be in line with finding no significant association between the overall microbial communities and the CIBDAI score. However, further studies are warranted to evaluate the intestinal microbiota of dogs with CIE in correlation to disease classification and the severity of microscopic intestinal disease.

Despite no differences in the global intestinal microbial communities, several bacterial taxa were found to be enriched in the two disease groups of dogs when applying LEfSe, a statistical tool to identify biomarkers between groups based on relative abundances (SEGATA et al. 2011). Similar to previous studies (XENOULIS et al. 2008, SUCHODOLSKI et al. 2010, SUCHODOLSKI et al. 2012a, MINAMOTO et al. 2015), abundances of Proteobacteria differed among dogs with CIE in our study. On the one hand, abundances of members of this phylum differed between both disease groups (e.g., unclassified genus of Neisseriaceae in the duodenum of dogs with IBD, *Bilophila* spp*.* in the duodenum of dogs with FRD). On the other hand, abundances of bacterial taxa within this phylum differed within each disease group and also based on the treatment status. Proteobacteria were found to be enriched, for example, in the duodenum of dogs with FRD (e.g., *Delftia* spp.) and in the colon of dogs with IBD (e.g., *Citrobacter* spp., *Burkholderia* spp.) before treatment when compared to their numbers after treatment. Finding Proteobacteria in large quantities is not surprising as they belong to the most abundant phyla in the gastrointestinal tract of healthy dogs (SUCHODOLSKI et al. 2008, XENOULIS et al. 2008, SUCHODOLSKI et al. 2009). However,

the increased presence in both the duodenum and colon of dogs with FRD and dogs with IBD before treatment might suggest a causative role in the development of CIE. One variant of a novel family of Burkholderiales, for example, was associated with perianal Crohn's disease in humans (SIM et al. 2010). *Burkholderia* spp. also represented a member of Proteobacteria that was more abundant in dogs with FRD and in dogs with IBD before treatment in this study. However, in the study by SIM et al. (2010), the disease-associated variant was identified in the ileal mucosa, whereas in the current study, *Burkholderia* spp. was detected in the colonic mucosa of dogs both with FRD and IBD. Also, lack of identification of the exact strain of *Burkholderia* spp. in the current study does not allow for further conclusions and leaves room for future investigations on the role of *Burkholderia* spp. in canine chronic enteropathies.

The genus *Bacteroides* spp. was abundant in the colon of dogs with FRD and also in dogs with IBD after treatment. Members of Bacteroidia have been identified more commonly in healthy dogs than in dogs diagnosed with IBD (XENOULIS et al. 2008, MINAMOTO et al. 2015, CASSMANN et al. 2016). Thus, our finding of increased abundances of *Bacteroides* spp. post-treatment might reflect a positive response to treatment and a trend towards amelioration of the disease. Conversely, certain strains of *Bacteroides* spp. also express virulence factors (e.g., production of a polysaccharide capsule, biofilm formation, modification of surface polysaccharides with resultant evasion of host immune system, expression of virulent proteases, and production of enterotoxins) (WEXLER 2007, REIS et al. 2014) and can potentially promote abscess formation via their polysaccharide capsule (WEXLER 2007). The latter proves to be a particular risk in human patients (WEXLER 2007) but – to the author's knowledge – this has not been reported to be a common clinical finding in dogs. Despite their potential virulence factors, it should be emphasized that *Bacteroides* spp. play a central role in the microbial degradation of carbohydrates (FLINT et al. 2012). Short-chain fatty acids, such as acetate, propionate, and butyrate, are essential factors for the host's intestinal health due to the provision of energy (NEISH 2009, SWANSON et al. 2011), regulation of intestinal motility (SUCHODOLSKI 2016), and anti-inflammatory properties (ARPAIA et al. 2013). Lastly, certain species of *Bacteroides* play a role in intestinal bile acid deconjugation and therefore are of additional benefit to the host (PAVLIDIS et al. 2015).Thus, altogether the overrepresentation of *Bacteroides* spp. in dogs with CIE after treatment in the current study leads the author to propose that *Bacteroides* spp. should be seen as "a friend" rather than "a foe". In addition, the quantitative identification of *Bacteroides* spp. might be a marker of the response to treatment. However, identification of the specific strains would be necessary for more definite conclusions.

4.2.2 Critical Appraisal of the Study's Protocol

A diagnosis of IBD requires chronic gastrointestinal signs, histologic evidence of mucosal inflammation, exclusion of any other underlying disorder that can mimic the clinical signs of IBD, and the need for anti-inflammatory and / or immunosuppressive treatment after failure to respond to appropriate therapeutic (e.g., antiparasitic, antimicrobial) treatment and at least three to four weeks of an elimination dietary trial (WASHABAU et al. 2010, SIMPSON and JERGENS 2011, ALLENSPACH et al. 2016, DANDRIEUX 2016). In the current study, the dietary trial was performed for 14 days and dogs that did not respond significantly within that period were assigned to the IBD group. Usually, the clinical response to an elimination diet is observed within a few days in dogs with FRD, but in some dogs it may take up to 14 days or even longer (up to three to four weeks) for a clinical improvement to be noticed (MARKS et al. 2002, ALLENSPACH et al. 2007, GASCHEN and MERCHANT 2011, ALLENSPACH et al. 2016). Therefore, some dogs with FRD might have been classified incorrectly as IBD in this study. However, the short duration of the dietary trial was chosen to ensure owner compliance. Because the majority of dogs with FRD respond within the chosen time frame of 14 days (MARKS et al. 2002, ALLENSPACH et al. 2007, GASCHEN and MERCHANT 2011, ALLENSPACH et al. 2016), it is the author's opinion that the benefit of owner compliance outweighs the risk of incorrect classification. A standardized antimicrobial trial was not included in the protocol of this study. While some studies have reported antibiotics to be effective for the treatment of chronic diarrhea (WESTERMARCK et al. 2005, KILPINEN et al. 2011, KILPINEN et al. 2014, KILPINEN et al. 2015), relapses of clinical signs after cessation of antimicrobial treatment are common (WESTERMARCK et al. 2005, ALLENSPACH et al. 2016). Thus, the true usefulness of antibiotics in the treatment of CIE has been questioned. In addition, the rapid increase of antimicrobial resistance in recent years argues against the standard practice of such treatment trials.

The endoscopic biopsy specimens used for this study were archived tissue samples that had been collected between 2006 and 2008 and were stored frozen until analysis. DNA is generally regarded as stable but it is still susceptible to hydrolysis, oxidation, and non-enzymatic methylation (LINDAHL 1993). Growing interest in the human microbiome, the Human Microbiome Project (TURNBAUGH et al. 2007), required further study to find the ideal technique to preserve DNA. This led to research undertakings on the optimal processing and storage conditions of DNA for subsequent microbiome analysis (GORZELAK et al. 2015, KLYMIUK et al. 2016). One study evaluating the microbiome of skin swabs after storage for different time periods revealed differences in the relative abundance of certain bacterial genera that predominate on the skin and also in the numbers of certain main phyla between different storage durations (KLYMIUK et al. 2016). Hence, an effect of DNA storage and processing on the results of the current study cannot be definitively excluded. Despite

efforts to standardize and improve microbiome analysis (SINHA et al. 2015), there are currently no guidelines about the processing of mucosal samples for subsequent microbiome analysis. In the current study, DNA was stored and handled to the best of our knowledge. DNA was extracted using the Mo Bio PowerSoil® DNA Isolation Kit. This method was also used in the Human Microbiome Project and is currently the most effective method of DNA extraction (WAGNER MACKENZIE et al. 2015). Thus, this method is currently recommended for extraction of DNA. In addition, further analysis of samples was performed at a highly respected laboratory in terms of microbiome analysis in companion animals (Gastrointestinal Laboratory, Texas A&M University, USA), minimizing unfavorable effects due to the handling of DNA.

The influence of diet on the intestinal microbial composition has been reported both in humans and in companion animals (SCOTT et al. 2013, PINNA et al. 2016, HERSTAD et al. 2017, LI et al. 2017). However, most of those studies revealing a dietary effect on the microbiome used diets with significant alterations in the composition of macronutrients, which does not compare to commonly used diets. Furthermore, one recent study showed that bacterial alpha diversity did not correlate with either dietary fat or protein intake in a population of dogs with IBD and control dogs (VÁZQUEZ-BAEZA et al. 2016), concluding that the disease effect is stronger than the diet's effect on the microbial composition. Further, to minimize the possibility of a delayed or persistent effect of the previous diet on the intestinal microbiota, dogs in this study would have had to be fed a standardized diet for several weeks before inclusion in the study. This, however, is very difficult to realize in a clinical trial. Taking the findings of VÁZQUEZ-BAEZA et al. (2016) into account, the author considers the variation among dogs, especially in terms of their previous diet, a realistic and valuable feature for a clinical patient population.

4.3 Important Aspects of the Evaluation of the Systemic Phospholipid Profile in Dogs with IBD and FRD

4.3.1 Further Discussion of the Results of the Phospholipidome Analysis

The phospholipid profile differed among samples dependent on the type of specimen (whole blood versus plasma), and among dogs based on the disease category and treatment status. The most relevant difference was found by principal component analysis (PCA) between whole blood and plasma samples. Phospholipids, and lipids in general, are essential constituents of cellular membranes (DE GIER and VAN DEENEN 1961, SCHROIT and ZWAAL 1991). Whole blood, which contains all blood components, has a higher cellularity

than plasma, the lipids of which are mainly comprised of lipoproteins. Thus, the observed difference likely results from the different cellular contents in the two types of specimens.

Altogether, treatment and disease category were found to be the most significant factors affecting the phospholipidome. As for the treatment status, a remarkable shift of phosphatidylcholine (PC) species was noticed from PC 38:4 (18:0 / 20:4) prior to treatment to the corresponding lysolipid PC 18:0 after treatment. Arachidonic acid (20:4) has been reported to be exclusively attached to the second carbon, also referred to as sn2-position, of the glycerol backbones of phospholipids (BEERMANN et al. 2005). The loss of arachidonic acid from the sn-2 position after initiation of treatment could potentially result from an activation of phospholipase A2, which releases arachidonic acid. Arachidonic acid, in turn, serves as a precursor for the synthesis of pro-inflammatory (e.g., prostaglandins and leukotrienes) and also anti-inflammatory mediators (e.g., lipoxins and resolvins) (KUEHL and EGAN 1980, SERHAN et al. 1984, BANNENBERG et al. 2005). The fact that the majority of dogs in our study showed considerable clinical improvement after the initiation of treatment might suggest such mechanisms (e.g., a loss of arachidonic acid in favor of anti-inflammatory mediators) to contribute to the amelioration of clinical signs. The treatment protocol in this study included an elimination diet based on fish, which was further enriched with dietary PUFAs. Several studies have evaluated the beneficial and anti-inflammatory effects of PUFAs of marine origin (SHORES et al. 2011, CALDER 2013, UNGARO et al. 2017, CHARPENTIER et al. 2018). PUFAs have been shown to reduce leucocyte chemotaxis and the expression of adhesion molecules, decrease the production of inflammatory cytokines, lower arachidonic acid-derived pro-inflammatory eicosanoids (e.g., prostaglandin $E_{2,}$ 4-series leukotrienes) in favor of anti-inflammatory eicosanoids (e.g., prostaglandin $E_{3,}$ 5-series leukotrienes), and decrease T cell reactivity (CALDER 2013, UNGARO et al. 2017). Furthermore, PUFAs can aid in the generation of additional anti-inflammatory mediators, such as resolvins and protectins (SERHAN et al. 2000, HONG et al. 2003, BANNENBERG et al. 2005). All of these beneficial characteristics of PUFAs support the hypothesis that the observed shift to lysoPC 18:0 after initiation of treatment reflects an increase in anti-inflammatory mediators, which might contribute to the improvement of clinical signs seen in the dogs with CIE after treatment.

Noteworthy is also the significant association of disease classification with the phospholipid profile observed in this study. A possible explanation for this finding could be the corresponding severity of the disease. Generally, clinical severity of canine CIE increases from FRD to IBD to IBD with PLE (ALLENSPACH et al. 2007, ALLENSPACH et al. 2016). This study found a significant effect of treatment on the phospholipidome in whole blood to be associated with the disease category, with the effect being largest for dogs diagnosed

with IBD with PLE. Hence, the loss of pro-inflammatory arachidonic acid might be greatest in those dogs with CIE that have the most severe clinical signs. Because the quantity of anti-inflammatory mediators required to modulate or counteract inflammation is likely higher the more severe the disease process, it might explain that the largest effect (e.g., loss of arachidonic acid and the change in the phospholipid profile) is present in those dogs with the most severe clinical disease.

The body condition score (BCS) only had a significant effect on the phospholipid profile in whole blood samples in PrComp2, and body weight (but not BCS) appeared to have some effect on the phospholipidome in plasma. However, both BCS and body weight only presented minor predictors of the phospholipid variance in this study, which is contrary to the results of previous studies. Those studies compared the lipidome of healthy and overweight dogs and identified a clear difference in both cholesterol concentration and lipoprotein profiles (JEUSETTE et al. 2005, MORI et al. 2011), particularly increased cholesterol and triglyceride concentrations in obese dogs (JEUSETTE et al. 2005). The low impact of BCS as well as body weight on the phospholipidome in the current study could be due to the fact that most of the dogs were assigned a similar and close to ideal BCS (BCS between 4/9 and 6/9) at their first visit. In addition, only three dogs had a BCS ≥ 7/9 and more advanced diagnostics to determine body fat (e.g., measured by dual-energy x-ray absorptiometry) were not performed in this study, rendering the evaluation of a potential effect of obesity impossible. Previous studies further reported age, gender, and breed to affect the lipoprotein concentrations of dogs (BARRIE et al. 1993, PASQUINI et al. 2008, MORI et al. 2011). While one study proposed more significant changes in plasma lipoprotein concentrations in older obese dogs (MORI et al. 2011), another study reported increased plasma concentrations of LDL-cholesterol in puppies and also increased cholesterol concentrations in certain breeds such as Rottweilers and Pyrenees Mountain dogs (PASQUINI et al. 2008). Different from those previous findings, neither gender nor breed had a significant effect on the lipid profile in the current study. However, it should be noted that despite not reaching statistical significance, the effect of breed was the most important effect on the phospholipidic variance on PrComp1 in whole blood specimens in the present study. A possible explanation for the discrepancy between our findings and those of previous studies could be the focus on different classes of lipids. While our study investigated primarily phospholipids, other studies mostly evaluated the plasma levels of cholesterol and lipoproteins.

4.3.2 Effect of Diets on the Systemic Lipid Profile

Similar to the effect of diet on the intestinal microbiota, a number of studies also suggest the diet composition to significantly affect the lipid profile and lipid metabolism (DOWNS et al.

1997, JEUSETTE et al. 2005, WRIGHT-RODGERS et al. 2005, PASQUINI et al. 2008). One study revealed that dogs receiving a diet with a high content of fish and fish by-products had the lowest serum cholesterol concentrations (PASQUINI et al. 2008). Another research group found that dietary modification to a high-protein low-energy diet resulted in a significant decrease in circulating cholesterol and triglyceride concentrations in obese dogs, and these beneficial effects on the plasma lipid profile were already present before any weight loss had occurred (JEUSETTE et al. 2005). Furthermore, diet composition has been reported to affect both phospholipid structure and characteristics of cellular membranes (e.g., lipid rafts, membrane fluidity), drawing attention to dietary phospholipids as a potential novel treatment option (MUELLER et al. 2004, GOLDBERG and KATZ 2007, MARION-LETELLIER et al. 2013). Overall, an influence of the pre-study diet on the pre-treatment systemic phospholipid profile of dogs is likely in this study. Feeding a standardized diet for several weeks before the dogs' enrollment in the study more likely would have avoided such a possible effect. Standardization of the diet, however, would have been very difficult, if not impossible, in the setting of a clinical trial.

4.4 Outlook: Strategies for Novel Treatment Options in Dogs with CIE

4.4.1 Prebiotics and Probiotics

The discovery of disease-associated alterations in the microbial composition led to further research to find novel treatment options with the goal of modulating the intestinal microbiome. Thus, the use of pre- and probiotics has become a focus of several investigations (SCHMITZ and SUCHODOLSKI 2016, WHITTEMORE and SUCHODOLSKI 2016). Probiotics are live microorganisms administered for their possible beneficial effects on the host, whereas prebiotics are dietary ingredients that are fermented by intestinal bacteria and can change the microbial composition and activity, again resulting in a benefit for the host (SCHMITZ and SUCHODOLSKI 2016). Synbiotics are mixtures of both pre- and probiotics (SCHMITZ and SUCHODOLSKI 2016). The exact mechanisms of how probiotics act to the benefit of the host are not fully understood, but probiotics have been shown to modulate the host's immune system (e.g., by their metabolites, cell wall components, and DNA), affect other microorganisms directly (e.g., through production of bacteriocins or competition for substances and / or space), and also to affect microbial and host products (e.g., by inactivation of bacterial toxins or detoxification of dietary components) (OELSCHLAEGER 2010). One study reported preventative effects of probiotics in antibiotic-associated diarrhea in humans (VIDELOCK and CREMONINI 2012). Short-term treatment with antibiotics can cause alterations in the fecal microbiota composition and an increase in antimicrobial resistance genes for up to several years in humans (JAKOBSSON et al. 2010). Further, the perturbation of the intestinal microbiota caused by an antibiotic agent has been

reported to outlast the period of antimicrobial administration in dogs (SUCHODOLSKI et al. 2009). Thus, the potential to prevent antibiotic-associated adverse effects poses a major advantage for the use of probiotics.

A large number of different probiotics is currently commercially available, but only four bacterial products have been investigated by the European Food Safety Authority for their efficient and harmless use in dogs: *Enterococcus faecium NCIMB 10415 E1705, Enterococcus faecium NCIMB 10415 E1707, Lactobacillus acidophilus DSM 13241 25*, and *Bifidobacterium sp. animalis* (SCHMITZ and SUCHODOLSKI 2016). Of these strains, only the two *Entercoccus faecium* strains have been considered reliably safe and efficient by the European Food Safety Authority (SCHMITZ and SUCHODOLSKI 2016). However, SCHMITZ et al. did not demonstrate any effect on clinical severity score, histologic lesion score, or the expression of inflammasome genes in dogs with CIE when administered the probiotic *Entercoccus faecium NCIMB 10415 E1707* (SCHMITZ et al. 2015a, SCHMITZ et al. 2015b), thus questioning the true efficacy of this product. In general, the number of studies evaluating and providing evidence of true efficiency of probiotic strains in the clinical setting is currently rather small, especially in the treatment of canine chronic enteropathies. One ex-vivo study showed favorable effects of a probiotic mixture of two *Lactobacillus acidophilus* strains and one *Lactobacillus johnsonii* strain on regulatory cytokines in dogs with CIE, which have the potential effect to ameliorate inflammation (SAUTER et al. 2005). A later in-vivo study of the same research group, however, showed that such effects of probiotics on inflammatory cytokines were inconsistent in dogs with FRD (SAUTER et al. 2006). This discrepancy emphasizes the need of well-designed and sufficiently powered clinical trials to allow for more definitive conclusions about the benefit of probiotics. A probiotic mixture with VSL#3 strains has been reported to show efficacy in the treatment of ulcerative colitis in humans (BIBILONI et al. 2005). VSL#3 is a probiotic product that contains four strains of *Lactobacilllus* spp. (*L. paracasei, L. plantarum, L. acidophilus, L. delbrueckii subsp. bulgaricus*), three strains of *Bifidobacterium* spp. (*B. longum, B. breve, B. infantis*), and one strain of *Streptococcus sulivarius subsp. thermophilus* (ROSSI et al. 2014, SCHMITZ and SUCHODOLSKI 2016). Recently, the use of VSL#3 strains was compared to a treatment protocol with prednisolone and metronidazole in dogs with IBD (ROSSI et al. 2014). That study showed effectiveness of the VSL#3 strains, with a decrease in clinical disease activity, histologic lesion score, and the number of intestinal $CD3^+$ T lymphocytes. The increase of a regulatory T cell marker ($FoxP3^+$) in the probiotic group further supports the existence of protective effects of VSL#3. Additionally, an increase in *Faecalibaterium* spp. was only observed in those dogs treated with VSL#3 (ROSSI et al. 2014). Because a decrease in *Faecalibacterium* spp. has been associated with intestinal dysbiosis (MIQUEL et al. 2013), an increase in this bacterial strain in the group of dogs treated with VSL#3 supports the

clinical use of VSL#3 strains in dogs with chronic enteropathies. It is noteworthy that, despite the finding that a decrease of *Faecalibacterium* spp. has been associated with intestinal dysbiosis (SOKOL et al. 2008, MIQUEL et al. 2013) and despite the proposed use of this bacterium as a probiotic in human medicine (SOKOL et al. 2008, MARTÍN et al. 2015), there is – to the author's knowledge – currently no study evaluating strains of *Faecalibacterium* spp. as probiotics in dogs.

In the current study, *Bacteroides* spp. presented at significant abundance in the colon of dogs with FRD and IBD after treatment. In addition to their possible virulent effects (WEXLER 2007), *Bacteroides* spp. have generally been recognized to play an important role for the host because of their involvement in carbohydrate degradation and bile acid deconjugation (FLINT et al. 2012, PAVLIDIS et al. 2015). Furthermore, *Bacteroides fragilis* has been shown to possess immunoregulatory properties (DENG et al. 2016) and to protect animals from experimental colitis (MAZMANIAN et al. 2008). Likewise, *Bacteroides uniformis* has been reported to have a higher prevalence in breast-fed infants with a lower risk to develop celiac disease compared to those with a higher genetic risk (SÁNCHEZ et al. 2011). The strain *B. uniformis CECT 7771* has further been reported to ameliorate metabolic and immune dysfunction induced by a high fat diet (GAUFFIN CANO et al. 2012). Because of all of these promising findings, *Bacteroides* spp. have recently attracted attention with regard to their value as a probiotic. However, to the author's knowledge, no study has assessed strains of *Bacteroides* spp. as probiotics in dogs to date. Since probiotic qualities of bacteria are strain specific (LIN et al. 2009, SCHMITZ and SUCHODOLSKI 2016), and the bacterial species was the lowest identified bacterial taxon in the current study, no definitive conclusions can be drawn from the high abundance of *Bacteroides* spp. after treatment in this study. However, considering all aspects outlined above, the author regards *Bacteroides* spp. and also *Faecalibacterium* spp. as promising bacteria that warrant further evaluation for their potential clinical utility as a probiotic.

One study demonstrated that probiotic strains of a synbiotic mixture (a blend of seven strains of lactic acid bacteria, fructooligosaccharides, and arabinogalactans), that was given to healthy dogs and cats, could be detected in the feces in almost all dogs and cats during the time of synbiotic administration, but neither before nor after the administration (GARCIA-MAZCORRO et al. 2011). Furthermore, despite a significant increase in the abundance of two of the probiotic strains at some time point during the administration of the synbiotic (*Enterococcus* spp., *Streptococcus* spp.), no changes in the major bacterial phyla were observed (GARCIA-MAZCORRO et al. 2011). These findings suggest that the administration of probiotics is safe but a sustained and major change of the microbiota composition cannot be achieved.

To conclude, probiotics may be a promising option in the prevention of antibiotic-associated diarrhea, and certain probiotic strains might be beneficial in the treatment of CIE. However, more studies are needed to fully understand the mutual reaction of probiotic bacteria and the host's distinct microbiome and to identify and further evaluate more specific bacterial strains that are able to affect the host in a beneficial but more sustained manner.

4.4.2 Fecal Microbiota Transplantation

The current lack of a lasting effect and the necessity to affect the intestinal microbial composition more extensively than has been achieved by single bacterial strains has drawn the attention to the concept of fecal microbial transplantation. Fecal microbiota transplantation (FMT), also referred to as fecal bacteriotherapy, is defined as *"administration of a solution of fecal matter from a donor into the intestinal tract of a recipient in order to directly change the recipient's microbial composition and confer a health benefit"* (GUPTA et al. 2016). Albeit being in its infancy, this treatment modality has gained importance in the treatment of recurrent *Clostridium difficile* infections in humans with a cure rate of nearly 90% (GOUGH et al. 2011, KASSAM et al. 2013, KELLY et al. 2015, GUPTA et al. 2016). In addition, FMT has recently been used in the treatment of human IBD with a clinical remission rate of approximately 36% and an overall good tolerance (COLMAN and RUBIN 2014). Sporadically, FMT has also been used in the treatment of irritable bowel syndrome (KELLY et al. 2015, GUPTA et al. 2016) and has been advocated for the treatment of obesity, metabolic syndrome, and diabetes mellitus (VRIEZE et al. 2012, KELLY et al. 2015, GUPTA et al. 2016). However, validated clinical data on these latter indications for FMT is limited rendering definite conclusions currently impossible.

To date, studies evaluating the use of FMT in companion animals are still scarce and are limited to anecdotal reports (CHAITMAN et al. 2016). In one study, three dogs with antibiotic-responsive diarrhea received FMT from the same donor dog resulting in a shift of the fecal microbial composition towards that of the donor and an improvement of the microbial diversity in all three dogs. However, a true clinical response was only seen in two dogs (GERBEC 2016). One case report of a dog with eosinophilic IBD revealed that the dog's fecal consistency had improved within 24 hours, and species richness of the fecal microbiota resembling the donor's microbiota had increased within two days after FMT (WEESE et al. 2013). Another study including eight dogs with refractory *Clostridium perfringens*-associated diarrhea reported an immediate resolution of diarrhea in all dogs and negative results on follow-up PCR panels for *Clostridium perfringens*-alpha toxin in 6 of 8 dogs after FMT (MURPHY et al. 2014). Furthermore, a current study on FMT in puppies with acute hemorrhagic diarrhea due to canine parvovirus enteritis showed that FMT in addition to

standard treatment yielded a faster resolution of diarrhea and a shorter time of patient hospitalization when compared to standard treatment alone. That study also reported FMT to be safe and well tolerated (PEREIRA et al. 2018). Thus, FMT appears to be a feasible and promising treatment option, especially in clinical cases where standard treatment protocols fail. Without doubt, larger-scale standardized prospective treatment trials are needed to investigate the true value of FMT and allow for general recommendations on the selection of donors, processing, and administration of FMT.

4.4.3 Health-Promoting Effects of Phospholipids and Polyunsaturated Fatty Acids

Phospholipids are amphiphilic lipids present in all cellular membranes of animals and plants (KÜLLENBERG et al. 2012). Glycerophospholipids represent the most prevalent phospholipids in biological membranes and are composed of a glycerol backbone with three carbons, fatty acids that are esterified to carbon 1 and 2, and a phosphate group with a hydrophilic residue (e.g., choline or inositol; resulting in phosphatidylcholine or phosphatidylinositol) esterified to carbon 3 (KÜLLENBERG et al. 2012, YAMASHITA et al. 2014). Glycerophospholipids found in food products such as dairy products, eggs, meat, seafood, and soybeans are classified as dietary glycerophospholipids (LORDAN et al. 2017). These dietary phospholipids usually have a saturated fatty acid on their first carbon (sn-1), while unsaturated fatty acids, such as oleic acid, arachidonic acid, or eicosapentaenoic acid (EPA), are located on the sn2-position. Because of the abundance of glycerophospholipids in biological membranes, their fatty acid composition has a major effect on membrane quality and stability (KÜLLENBERG et al. 2012). Furthermore, it has been shown that fatty acids provided by dietary phospholipids can be incorporated into cellular membranes (TAYLOR et al. 2010). Thus, dietary glycerophospholipids are able to alter the fatty acid composition of biological membranes, allowing for their ability to modulate cellular functions (e.g., signaling and transport) and membrane-bound enzymes (KÜLLENBERG et al. 2012, LORDAN et al. 2017). This ability is likely one reason for the positive effects on health that have been attributed to dietary phospholipids in several diseases in humans (KÜLLENBERG et al. 2012). Dietary phospholipids, for example, have been shown to lower cholesterol levels (COHN et al. 2010) and to be effective adjuvants in the treatment of liver disease (POONGOTHAI et al. 2005, GUNDERMANN et al. 2011, GUNDERMANN et al. 2016). Furthermore, phosphatidylcholine derivatives in cheese and yogurt have been reported to have antithrombotic, anti-inflammatory, and cardioprotective properties (TSOROTIOTI et al. 2014, MEGALEMOU et al. 2017). Another group of researchers also showed that soybean phosphatidylcholine can reduce the risk of cardiovascular disease by decreasing plasma concentrations of homocysteine, which in high concentration is suspected to be associated with a higher risk of cardiovascular disease (OLTHOF et al. 2005). Several studies further

revealed beneficial effects of phospholipids in patients diagnosed with cancer. Those effects include the potential to inhibit tumor growth and metastasis (HOSSAIN et al. 2008, SAKAKIMA et al. 2009, JANTSCHEFF et al. 2011, KÜLLENBERG et al. 2012). Another study demonstrated that oral supplementation of phospholipids for several weeks was associated with a stabilization of body weight with improved appetite and quality of life in cancer patients (TAYLOR et al. 2010). In addition, the glycerophospholipid composition of neural membranes has been shown to be altered in neurological disorders and aging, with the amount of phosphatidylcholine, phosphatidylserine, and PUFAs in the brain being of greatest importance (FAROOQUI et al. 2000, KÜLLENBERG et al. 2012). Hence, several studies have investigated the potential benefits of administering phospholipids to patients with neurological disorders. Those studies led to the current consensus that supplementation of phospholipids, particularly of phosphatidylserine, can significantly improve cognition, memory function, and also the general mood (LOUIS-SYLVESTRE 1999, BENTON et al. 2001, RICHTER et al. 2013). Thus, phospholipids hold promising health-promoting effects, ostensibly without severe adverse effects. The exact mechanisms of these beneficial effects are currently not completely understood, but some effects can be attributed to fatty acids, particularly polyunsaturated fatty acids, delivered by phospholipids. As outlined above, phospholipids primarily contain PUFAs on their sn2-position, and can be cleaved from that position and made available for further conversion and cellular processes (KÜLLENBERG et al. 2012, LORDAN et al. 2017).

Polyunsaturated fatty acids have been recognized to play a role in inflammatory processes, with the omega-6 PUFA arachidonic acid and the omega-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) being of vital importance (CALDER 2013). While eicosanoids derived from arachidonic acid (e.g., several prostaglandins, thromboxane A_2, leukotriene E_4) are mainly pro-inflammatory, eicosanoids generated from EPA and DHA (e.g., resolvins, leukotriene E_5, protectins) are usually less immunostimulatory and have rather anti-inflammatory properties. Besides competing against arachidonic acid metabolites, the latter substances also impede leukocyte chemotaxis, interfere with endothelial adhesion, reduce cytokine synthesis, and affect T cell function (CALDER 2010, BARBALHO et al. 2016, UNGARO et al. 2017). Thus, PUFAs have been widely used as dietary supplements in both human and veterinary medicine as adjuvant treatment modalities in various disorders (SIMOPOULOS 2002, BAUER 2011, CALDER 2013). In human medicine, attention has recently also been turned to possible benefits of omega-3 PUFAs in patients with IBD, and several studies support their clinical use (CALDER 2013, BARBALHO et al. 2016, UNGARO et al. 2017, CHARPENTIER et al. 2018). One study, for example, showed that omega-3 PUFAs are protective against oxidative stress in patients with ulcerative colitis (BARBOSA et al. 2003). Another study found that a diet high in DHA might reduce the risk to develop

Crohn's disease (CHAN et al. 2014). On the other hand, a number of studies have failed to confirm a true clinical benefit of omega-3 fatty acids in patients with IBD (CALDER 2013, BARBALHO et al. 2016), such as one study that could not identify a beneficial effect of omega-3 PUFAs on the risk of relapse of active Crohn's disease (FEAGAN et al. 2008). Thus, evidence of a true therapeutic benefit of omega-3 PUFAs in human IBD is still lacking.

In veterinary medicine, PUFAs are commonly administered in the treatment of cardiovascular disease, renal disease, ostheoarthritis, and canine atopic dermatitis (MUELLER et al. 2004, OLIVRY et al. 2010, BAUER 2011, BAUER 2016). When used to treat atopic dermatitis, PUFAs seem to also have a cyclosporine-sparing effect alongside their anti-inflammatory properties (MÜLLER et al. 2016). Furthermore, when highly enriched in the diet, DHA might favorably affect cognitive, retinal, and immunologic functions in young dogs (ZICKER et al. 2012). However, only few studies have investigated the potential benefits of PUFAs in the treatment of canine chronic enteropathies to date. One study reported significantly altered intestinal mucosal mRNA levels of membrane proteins involved in cholesterol homeostasis after feeding dogs a diet supplemented with PUFAs. These alterations in mucosal gene expression have been suggested to improve cellular cholesterol metabolism, thus having a positive effect in dogs with IBD (ONTSOUKA et al. 2010). The same group of researchers also reported significant differences in the mucosal abundance of genes regulating fatty acid uptake in dogs fed an omega-3 PUFA-enriched diet. These differences, again, suggest a benefit in dogs with CIE, especially for those diagnosed with FRD (ONTSOUKA et al. 2012). Therefore, both studies support the addition of omega-3 PUFAs to the treatment plan of dogs with CIE. However, PUFAs can also have adverse effects, for instance, by altering platelet function, causing diarrhea and vomiting, and impairing immune function (LENOX and BAUER 2013). In the present study, the PUFA-enriched diet was well-tolerated with no evidence of side effects. Nonetheless, the possibility of a sole effect of supplemental PUFAs on the phospholipid profile and the dogs' clinical improvement cannot be assessed based on the results of this study because of the lack of a control group of dogs with CIE receiving the same treatment but short of PUFAs. Thus, no conclusion on the particular benefit of PUFAs in the treatment of canine CIE can be drawn from the findings of this study. Additional research is warranted to further explore the potential benefit of administering omega-3 PUFAs to dogs with CIE.

4.5 Conclusions

This study identified some differences in individual bacterial taxa both between the disease groups of dogs with IBD and FRD and also with regard to the treatment status. The relevance of these bacterial groups in the pathogenesis of IBD and FRD is currently unknown. Further larger-scale studies are warranted to shed more light on the role of the intestinal microbiota in the pathophysiology of different CIE forms and to evaluate their potential therapeutic benefit. In the author's opinion, especially *Bacteroides* spp. should be a focus of future studies and can potentially serve as a marker of treatment response.

This study further detected significant variances in the systemic phospholipid profiles of dogs with IBD and dogs with FRD. These variances were predominantly associated with the type of specimen used, clinical disease severity, and treatment status. After initiation of treatment, a shift of phospholipid species from phosphatidylcholine PC 38:4 (18:0 / 20:4) towards lysophosphatidylcholine 18:0 was identified, which likely represents a loss of arachidonic acid. This loss of arachidonic acid likely happens in favor of anti-inflammatory mediators contributing to the amelioration of clinical signs. Thus, the loss of arachidonic acid might indicate disease amelioration. Future studies should further investigate the role of lipids in the pathophysiology of IBD and FRD.

The search for novel extended-effect treatment options has drawn attention to the modification of the intestinal microbiota and the administration of dietary phospholipids. Preliminary studies revealed promising results on the use of the probiotic mixture VSL#3, FMT, and dietary phospholipids, specifically PUFAs, in the treatment of canine CIE. Again, more standardized studies are needed in order to further evaluate the safety and true clinical efficacy of these therapeutic approaches.

In conclusion, this study aimed to assess both the intestinal mucosal microbiota and the systemic phospholipid profile in dogs diagnosed with IBD and FRD, and to evaluate potential differences associated with the treatment status. Though not a large number of differentiating bacterial species and phospholipids was detected, this study revealed some important factors and differences in the mucosal microbiota and the phospholipid profile. This study showed an increase of *Bacteroides* spp. and a shift towards lysophosphatidylcholine 18:0 – with a loss of arachidonic acid – after treatment. Both findings hold potential to serve as indicators of disease amelioration and as indicators of treatment response. This study should be considered a pilot study, encouraging future large-scale studies to confirm our findings and to further our understanding of the role of the intestinal microbiota and systemic phospholipids in the pathogenesis and treatment of canine CIE.

5 SUMMARY

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Differences in the Intestinal Microbiome and Lipidome of Dogs Diagnosed with Idiopathic Inflammatory Bowel Disease or Food-Responsive Diarrhea before and after the Induction Phase of Treatment

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Keywords: mucosal microbiota, systemic phospholipids, chronic inflammatory enteropathies, treatment, duodenum, colon

Background: Idiopathic inflammatory bowel disease (IBD) and food-responsive diarrhea (FRD) are common categories of chronic inflammatory enteropathy (CIE) in dogs. The intestinal microbiota is considered a major contributing factor to the pathogenesis of IBD. Intestinal dysbiosis has been identified in dogs with IBD, but only little information is available on differences in the mucosal microbiota of dogs diagnosed with IBD and FRD. In humans, dyslipidemia has also been described in patients with IBD. However, studies on the lipid profile in dogs with IBD or FRD are currently lacking.

Objectives: This is the first study to (1) compare the intestinal mucosal microbiota of dogs with IBD and FRD in the duodenum and the colon, (2) evaluate differences in the mucosal microbiota of each dog before and after the induction phase of treatment, and (3) evaluate the systemic phospholipid profile of dogs with IBD or FRD also both prior to and after the induction phase of treatment.

Materials and Methods: Duodenal and colonic mucosal biopsies from 24 dogs with CIE (15 FRD, 9 IBD) and EDTA-plasma and whole blood from 32 dogs (16 FRD, 16 IBD) were retrieved from a former study on canine CIE. All client-owned dogs were prospectively enrolled in the study. All dogs received a standardized diagnostic work-up and treatment including a dietary trial. Dogs that responded to the elimination diet within 14 days were classified as FRD; the remaining dogs requiring additional immunosuppressant treatment were classified as IBD. Biopsy specimens of duodenum and colon were obtained endoscopically both before and after standard therapy. DNA was extracted from these biopsies and the intestinal mucosal microbiota of the duodenum and colon were evaluated by Illumina sequencing of the bacterial 16S rRNA gene. The phospholipids in whole blood and EDTA plasma, collected both before and after treatment, were analyzed by hydrophilic interaction liquid chromatography (HILIC). Differences in the composition were statistically

assessed by alpha diversity indices, principal coordinate analysis, analysis of similarities (ANOSIM) and linear discriminant (LDA) analysis effect size (LEfSe) for the microbiota and by principal component analysis (PCA), analysis of variance (ANOVA) and random forest analysis for the phospholipid profile.

Results: All differences in the microbial composition and phospholipid profile described below are statistically confirmed with significance set at p-value < 0.05 or LDA score > 2.0. No difference in the global bacterial composition was identified neither between the two disease groups of dogs nor with the treatment status. However, abundances of several bacterial taxa varied between disease groups and also with the treatment status. When comparing disease groups, an unclassified genus of Neisseriaceae was more abundant in the duodenum in the IBD group, whereas *Bilophila* spp. occurred more frequently in the duodenum of the FRD group. Comparison before and after treatment revealed *Enterococcus* spp., *Corynebacterium* spp. and Proteobacteria to be enriched in the duodenum of FRD dogs before treatment. *Bacteroides* spp. was more abundant in the colon in the FRD group post-treatment. In the IBD dogs, Unclassified_Neisseriaceae was more abundant in the duodenum and mainly Proteobacteria (*Burkholderia* spp., *Citrobacter* spp.) in the colon prior to treatment. *Bacteroides* spp. was significantly more abundant in the colon after treatment.

The phospholipidome differed dependent on the type of specimen (whole blood vs. plasma). In addition, treatment and disease severity presented the most significant factors determining the variance of the phospholipid profile. An increase in lysolipids and a significant shift of the phosphatidylcholine (PC) species from PC 38:4 (18:0 / 20:4) to mainly lysophosphatidylcholine 18:0 was observed after treatment.

Conclusions: Some differences in individual bacterial taxa were identified both between disease groups of dogs and with regard to treatment status. The role of these bacterial groups in the pathogenesis of IBD and FRD is still unknown. However, *Bacteroides* spp. might be of importance, and this species could potentially serve as marker of treatment response. Furthermore, significant variances were identified in the phospholipid profiles of dogs with IBD and FRD, which were particularly associated with the type of specimen used, disease severity, and treatment status. These alterations in the phospholipid profile could potentially aid in monitoring the response to treatment. Subsequently, specific modulation of the intestinal microbiota and the phospholipid profile might also present novel therapeutic strategies in dogs with CIE.

6 ZUSAMMENFASSUNG

Katja Kalenyak

Unterschiede im intestinalen Mikrobiom und im Fettstoffwechsel bei Hunden mit idiopathischer Inflammatory Bowel Disease und Futter-responsiver Diarrhoe vor und nach Induktionsphase der Therapie

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Schlüsselwörter: Mukosale Mikrobiota, systemische Phospholipide, chronisch-entzündliche Enteropathien, Therapie, Duodenum, Kolon

Einleitung: Die idiopathische Inflammatory Bowel Disease (IBD) und die Futter-responsive Diarrhoe (FRD) sind häufige Formen der chronisch-entzündlichen Enteropathie (CIE) beim Hund. Die intestinale Mikrobiota wird als ein wichtiger Faktor in der Pathogenese der IBD angesehen. Eine intestinale Dysbiose wurde bei Hunden mit IBD nachgewiesen, allerdings liegen bisher nur wenig Informationen zu Unterschieden in der mukosalen Mikrobiota bei Hunden mit IBD und FRD vor. In der Humanmedizin ist zudem eine Dyslipidämie bei an IBD erkrankten Patienten beschrieben. Bisher gibt es allerdings noch keine Studien über das Lipidprofil bei Hunden mit IBD und FRD.

Zielstellung: Diese Studie vergleicht erstmals (1) die intestinale mukosale Mikrobiota bei Hunden mit IBD und FRD im Duodenum und im Kolon, (2) untersucht zusätzlich Unterschiede in der mukosalen Mikrobiota bei jedem Hund vor und nach der Induktionsphase der Therapie und (3) evaluiert das systemische Phospholipidprofil von Hunden mit IBD oder FRD ebenfalls sowohl vor als auch nach Therapie.

Material und Methoden: Archivierte Mukosabiopsien von Duodenum und Kolon von 24 Hunden mit CIE (15 FRD, 9 IBD) und EDTA-Plasma und Vollblut von 32 Hunden (16 FRD, 16 IBD) wurden aus einer früheren Studie über CIE übernommen. Alle Privathunde wurden ursprünglich prospektiv in die Studie eingeschlossen. Bei allen wurde eine standardisierte diagnostische Abklärung und Therapie inklusive einer Eliminationsdiät durchgeführt. Hunde, die innerhalb von 14 Tagen klinisch auf die Eliminationsdiät ansprachen, wurden als FRD klassifiziert. Die übrigen Hunde, die eine zusätzliche immunsuppressive Therapie benötigten, wurden als IBD-Patienten eingestuft. Biopsien von Duodenum und Kolon wurden sowohl vor als auch nach der standardisierten Therapie endoskopisch entnommen. DNA wurde aus diesen Biopsien extrahiert und die intestinale mukosale Mikrobiota mit Hilfe von Illumina-Sequenzierung des bakteriellen 16S rRNA Gens ausgewertet. Die Phospholipide in Vollblut

und EDTA-Plasma, welche ebenfalls jeweils vor und nach Therapie abgenommen wurden, wurden mit hydrophiler Interaktions-*Flüssigchromatographie* (HILIC) analysiert. Unterschiede in der Zusammensetzung wurden für die Mikrobiota mit Hilfe von Alpha-Diversität Indizes, Hauptkoordinatenanalyse, Ähnlichkeitsanalyse (ANOSIM) und lineare Diskriminanzanalyse (LDA) Effektgröße (LEfSe) und für das Phospholipidprofil mit Hauptkomponentenanalyse (PCA), Varianzanalyse (ANOVA) und Random Forest Analyse ermittelt.

Ergebnisse: Alle nachfolgend beschriebenen Unterschiede der Mikrobiota und des Phospholipidprofils sind mit einer Signifikanz bei p-Wert < 0,05 oder LDA-Wert > 2,0 statistisch belegt. Es wurden weder zwischen den zwei Krankheitsgruppen noch zwischen den Behandlungszeitpunkten Unterschiede in der globalen mukosalen bakteriellen Zusammensetzung gefunden. Allerdings waren einige bakterielle Taxa in den Krankheitsgruppen und in Hinblick auf den Behandlungsstatus unterschiedlich vorhanden. Beim Vergleich der Krankheitsgruppen war in der IBD-Gruppe eine unklassifizierte Gattung der Neisseriaceae im Duodenum stärker vorhanden, wohingegen *Bilophila* spp. im Duodenum der FRD Gruppe verstärkt vorkam. Der Vergleich vor und nach Therapie zeigte, dass *Enterococcus* spp., *Corynebacterium* spp. und Proteobakterien im Duodenum von Hunden mit FRD vor Therapie vermehrt vorkamen. Bei Hunden mit IBD waren vor Therapie eine unklassifizierte Gattung der Neisseriaceae im Duodenum und insbesondere Proteobakterien (*Burkholderia* spp.*, Citrobacter* spp.) im Kolon differenziell vorhanden. Nach Therapie kam *Bacteroides* spp*.* im Kolon sowohl von Hunden mit FRD als auch von Hunden mit IBD vermehrt vor.
Das Phospholipidom differierte in Abhängigkeit von der Blutprobe (Vollblut vs. Plasma). Zusätzlich stellten Therapie und Schweregrad der Erkrankung die wichtigsten Einflussfaktoren auf die Variation im Phospholipidprofil dar. Nach Therapie wurde ein Anstieg an Lysolipiden und eine signifikante Verschiebung der Spezies Phosphatidylcholin (PC) von PC 38:4 (18:0 / 20:4) zu hauptsächlich Lysophosphatidylcholin 18:0 beobachtet.

Schlussfolgerungen: Sowohl zwischen Krankheitsgruppen als auch im Hinblick auf den Behandlungsstatus konnten einige Unterschiede in einzelnen bakteriellen Taxa nachgewiesen werden. Die Bedeutung dieser Bakteriengruppen in der Pathogenese der IBD und FRD bleibt leider weiterhin ungeklärt. Jedoch schien *Bacteroides* spp*.* von großer Bedeutung zu sein, da diese Bakterienart möglicherweise als Marker für einen Behandlungserfolg dienen könnte. Zusätzlich wurden signifikante Varianzen in den Phospholipidprofilen von Hunden mit IBD und FRD festgestellt, welche abhängig waren von der Blutprobenart, dem Schweregrad der Erkrankung und dem Behandlungsstatus. Diese Varianzen im Phospholipidprofil könnten bei der Überwachung des Therapieerfolgs hilfreich sein. Insgesamt kann eine gezielte Beeinflussung der intestinalen Mikrobiota und des Phospholipidprofils auch einen neuen Therapieansatz für Hunde mit CIE darstellen.

7 REFERENCES

Abba C, Mussa PP, Vercelli A, Raviri G. Essential fatty acids supplementation in different-stage atopic dogs fed on a controlled diet. J Anim Physiol Anim Nutr (Berl). 2005;89(3-6):203-7.

Agouridis AP, Elisaf M, Milionis HJ. An overview of lipid abnormalities in patients with inflammatory bowel disease. Ann Gastroenterol. 2011;24(3):181-7.

Allenspach K, Rüfenacht S, Sauter S, Grone A, Steffan J, Strehlau G, Gaschen F. Pharmacokinetics and clinical efficacy of cyclosporine treatment of dogs with steroid-refractory inflammatory bowel disease. J Vet Intern Med. 2006;20(2):239-44.

Allenspach K, Wieland B, Gröne A, Gaschen F. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. J Vet Intern Med. 2007;21(4):700-8.

Allenspach K. Diseases of the Large Intestine. In: Ettinger SJ, Feldmann EC, editors. Textbook of Veterinary Internal Medicine. 7th ed. St. Louis, Missouri, USA: Saunders Elsevier; 2010a. p. 1573-94.

Allenspach K, House A, Smith K, McNeill FM, Hendricks A, Elson-Riggins J, Riddle A, Steiner JM, Werling D, Garden OA, Catchpole B, Suchodolski JS. Evaluation of mucosal bacteria and histopathology, clinical disease activity and expression of Toll-like receptors in German shepherd dogs with chronic enteropathies. Vet Microbiol. 2010b;146(3-4):326-35.

Allenspach K, Gaschen FP. Erkrankungen des Dünndarms. In: Steiner JM, editor. Gastroenterologie bei Hund und Katze: Klinik-Diagnostik-Therapie. Hannover, Germany: Schlütersche 2011. p. 193-207.

Allenspach K, Culverwell C, Chan D. Long-term outcome in dogs with chronic enteropathies: 203 cases. Vet Rec. 2016;178(15):368.

AlShawaqfeh MK, Wajid B, Minamoto Y, Markel M, Lidbury JA, Steiner JM, Serpedin E, Suchodolski JS. A dysbiosis index to assess microbial changes in fecal samples of dogs with chronic inflammatory enteropathy. FEMS Microbiol Ecol. 2017;93(11).

Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY. Metabolites produced by commensal bacteria promote peripheral regulatory T cell generation. Nature. 2013;504(7480):451-5.

Astarita G, Ollero M. Lipidomics: an evolving discipline in molecular sciences. Int. J. Mol. Sci. 2015;16(4):7748-52.

Bannenberg GL, Chiang N, Ariel A, Arita M, Tjonahen E, Gotlinger KH, Hong S, Serhan CN. Molecular circuits of resolution: formation and actions of resolvins and protectins. J Immunol. 2005;174(7):4345-55.

Barbalho SM, Goulart RdA, Quesada K, Bechara MD, de Carvalho AdCA. Inflammatory bowel disease: can omega-3 fatty acids really help? Ann Gastroenterol 2016;29(1):37-43.

Barbosa DS, Cecchini R, El Kadri MZ, Rodriguez MA, Burini RC, Dichi I. Decreased oxidative stress in patients with ulcerative colitis supplemented with fish oil omega-3 fatty acids. Nutrition. 2003;19(10):837-42.

Barrie J, Watson TDG, Stear MJ, Nash AS. Plasma cholesterol and lipoprotein concentrations in the dog: The effects of age, breed, gender and endocrine disease. J Small Anim Pract. 1993;34(10):507-12.

Batt RM, Barnes A, Rutgers HC, Carter SD. Relative IgA deficiency and small intestinal bacterial overgrowth in German shepherd dogs. Res Vet Sci. 1991;50(1):106-11.

Bauer JE. Therapeutic use of fish oils in companion animals. J Am Vet Med Assoc. 2011;239(11):1441-51.

Bauer JE. The essential nature of dietary omega-3 fatty acids in dogs. J Am Vet Med Assoc. 2016;249(11):1267-72.

Beermann C, Möbius M, Winterling N, Schmitt JJ, Boehm G. sn-Position determination of phospholipid-linked fatty acids derived from erythrocytes by liquid chromatography electrospray ionization ion-trap mass spectrometry. Lipids. 2005;40(2):211-8.

Behling-Kelly E. Serum lipoprotein changes in dogs with renal disease. J Vet Intern Med. 2014;28(6):1692-8.

Beilby J. Definition of Metabolic Syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association Conference on Scientific Issues Related to Definition. Clin Biochem Rev. 2004;25(3):195-8.

Benton D, Donohoe R, Sillance B, Nabb S. The influence of phosphatidylserine supplementation on mood and heart rate when faced with an acute stressor. Nutr Neurosci. 2001;4(3):169-78.

Berridge MJ. Inositol trisphosphate and calcium signalling. Nature. 1993;361(6410):315-25.

Bibiloni R, Fedorak RN, Tannock GW, Madsen KL, Gionchetti P, Campieri M, De Simone C, Sartor RB. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. Am J Gastroenterol. 2005;100(7):1539-46.

Breitschwerdt EB, Halliwell WH, Foley CW, Stark DR, Corwin LA. A hereditary diarrhetic syndrome in the Basenji characterized by malabsorption, protein losing enteropathy and hypergammaglobulinemia. J Am Anim Hosp Assoc. 1980;16(4):551-60.

Burgener IA, König A, Allenspach K, Sauter SN, Boisclair J, Doherr MG, Jungi TW. Upregulation of toll-like receptors in chronic enteropathies in dogs. J Vet Intern Med. 2008;22(3):553-60.

Burrows C. Canine hemorrhagic gastroenteritis. J Am Anim Hosp Assoc. 1977;13:451-8.

Calder PC. Omega-3 fatty acids and inflammatory processes. Nutrients. 2010;2(3):355-74.

Calder PC. Omega-3 polyunsaturated fatty acids and inflammatory processes: nutrition or pharmacology? Br J Clin Pharmacol. 2013;75(3):645-62.

Cao XY, Dong M, Shen JZ, Wu BB, Wu CM, Du XD, Wang Z, Qi YT, Li BY. Tilmicosin and tylosin have anti-inflammatory properties via modulation of COX-2 and iNOS gene expression and production of cytokines in LPS-induced macrophages and monocytes. Int J Antimicrob Agents. 2006;27(5):431-8.

Cassmann E, White R, Atherly T, Wang C, Sun Y, Khoda S, Mosher C, Ackermann M, Jergens A. Alterations of the Ileal and Colonic Mucosal Microbiota in Canine Chronic Enteropathies. PLoS ONE. 2016;11(2):e0147321.

Chaitman J, Jergens AE, Gaschen F, Garcia-Mazcorro JF, Marks SL, Marroquin-Cardona AG, Richter K, Rossi G, Suchodolski JS, Weese JS. Commentary on key aspects of fecal microbiota transplantation in small animal practice. Vet Med (Auckl). 2016;7:71-4.

Chan SS, Luben R, Olsen A, Tjonneland A, Kaaks R, Lindgren S, Grip O, Bergmann MM, Boeing H, Hallmans G, Karling P, Overvad K, Veno SK, van Schaik F, Bueno-de-Mesquita B, Oldenburg B, Khaw KT, Riboli E, Hart AR. Association between high dietary intake of the n-3 polyunsaturated fatty acid docosahexaenoic acid and reduced risk of Crohn's disease. Aliment Pharmacol Ther. 2014;39(8):834-42.

Charpentier C, Chan R, Salameh E, Mbodji K, Ueno A, Coëffier M, Guérin C, Ghosh S, Savoye G, Marion-Letellier R. Dietary n-3 PUFA may attenuate experimental colitis. Mediators Inflamm. 2018;2018:8430614.

Cohn JS, Kamili A, Wat E, Chung RW, Tandy S. Dietary phospholipids and intestinal cholesterol absorption. Nutrients. 2010;2(2):116-27.

Colman RJ, Rubin DT. Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis. J Crohns Colitis. 2014;8(12):1569-81.

Craven M, Simpson JW, Ridyard AE, Chandler ML. Canine inflammatory bowel disease: retrospective analysis of diagnosis and outcome in 80 cases (1995-2002). J Small Anim Pract. 2004;45(7):336-42.

Cutler RG, Kelly J, Storie K, Pedersen WA, Tammara A, Hatanpaa K, Troncoso JC, Mattson MP. Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. Proc Natl Acad Sci USA. 2004;101(7):2070-5.

Dandrieux JR, Noble P-JM, Scase TJ, Cripps PJ, German AJ. Comparison of a chlorambucil-prednisolone combination with an azathioprine-prednisolone combination for treatment of chronic enteropathy with concurrent protein-losing enteropathy in dogs: 27 cases (2007–2010). J Am Vet Med Assoc. 2013;242(12):1705-14.

Dandrieux JR. Inflammatory bowel disease versus chronic enteropathy in dogs: are they one and the same? J Small Anim Pract. 2016;57(11):589-99.

Davis CP, Cleven D, Balish E, Yale CE. Bacterial association in the gastrointestinal tract of beagle dogs. Appl Environ Microbiol. 1977;34(2):194-206.

Day MJ. The canine model of dietary hypersensitivity. Proc Nutr Soc. 2005;64(4):458-64.

Day MJ, Bilzer T, Mansell J, Wilcock B, Hall EJ, Jergens A, Minami T, Willard M, Washabau R. Histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from the dog and cat: a report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group. J Comp Pathol. 2008;138 Suppl 1:S1-43.

De Gier J, Van Deenen LLM. Some lipid characteristics of red cell membranes of various animal species. Biochim Biophys Acta. 1961;49(2):286-96.

Delles EK, Willard MD, Simpson RB, Fossum TW, Slater M, Kolp D, Lees GE, Helman R, Reinhart G. Comparison of species and numbers of bacteria in concurrently cultured samples of proximal small intestinal fluid and endoscopically obtained duodenal mucosa in dogs with intestinal bacterial overgrowth. Am J Vet Res. 1994;55(7):957-64.

Deng H, Li Z, Tan Y, Guo Z, Liu Y, Wang Y, Yuan Y, Yang R, Bi Y, Bai Y, Zhi F. A novel strain of *Bacteroides fragilis* enhances phagocytosis and polarises M1 macrophages. Sci Rep. 2016;6:29401.

Dill AL, Ifa DR, Manicke NE, Costa AB, Ramos-Vara JA, Knapp DW, Cooks RG. Lipid profiles of canine invasive transitional cell carcinoma of the urinary bladder and adjacent normal tissue by desorption electrospray ionization imaging mass spectrometry. Anal Chem. 2009;81(21):8758-64.

Downs LG, Bolton CH, Crispin SM, Wills JM. Plasma lipoprotein lipids in five different breeds of dogs. Res Vet Sci. 1993;54(1):63-7.

Downs LG, Crispin SM, LeGrande-Defretin V, Perez-Camargo G, McCappin T, Bolton CH. The effect of dietary changes on plasma lipids and lipoproteins of six Labrador retrievers. Res Vet Sci. 1997;63(2):175-81.

Duboc H, Rajca S, Rainteau D, Benarous D, Maubert M-A, Quervain E, Thomas G, Barbu V, Humbert L, Despras G, Bridonneau C, Dumetz F, Grill J-P, Masliah J, Beaugerie L, Cosnes J, Chazouillères O, Poupon R, Wolf C, Mallet J-M, Langella P, Trugnan G, Sokol H, Seksik P. Connecting dysbiosis, bile-acid dysmetabolism and gut inflammation in inflammatory bowel diseases. Gut. 2013;62(4):531.

Erdmann C, Heilmann RM. Diagnostic and therapeutic approach to chronic inflammatory enteropathies in dogs. Tierarztl Prax Ausg K Kleintiere Heimtiere. 2017;45(5):317-27.

Fahy E, Subramaniam S, Brown HA, Glass CK, Merrill AH, Jr., Murphy RC, Raetz CR, Russell DW, Seyama Y, Shaw W, Shimizu T, Spener F, van Meer G, VanNieuwenhze MS, White SH, Witztum JL, Dennis EA. A comprehensive classification system for lipids. J Lipid Res. 2005;46(5):839-62.

Fahy E, Subramaniam S, Murphy RC, Nishijima M, Raetz CR, Shimizu T, Spener F, van Meer G, Wakelam MJ, Dennis EA. Update of the LIPID MAPS comprehensive classification system for lipids. J Lipid Res. 2009;50 Suppl(Supplement):S9-14.

Fan F, Mundra PA, Fang L, Galvin A, Moore XL, Weir JM, Wong G, White DA, Chin-Dusting J, Sparrow MP. Lipidomic profiling in inflammatory bowel disease: comparison between ulcerative colitis and Crohn's disease. Inflamm Bowel Dis. 2015;21(7):1511-8.

Farooqui AA, Horrocks LA, Farooqui T. Glycerophospholipids in brain: their metabolism, incorporation into membranes, functions, and involvement in neurological disorders. Chem Phys Lipids. 2000;106(1):1-29.

Feagan BG, Sandborn WJ, Mittmann U, Bar-Meir S, D'Haens G, Bradette M, Cohen A, Dallaire C, Ponich TP, McDonald JW, Hebuterne X, Pare P, Klvana P, Niv Y, Ardizzone S, Alexeeva O, Rostom A, Kiudelis G, Spleiss J, Gilgen D, Vandervoort MK, Wong CJ, Zou GY, Donner A, Rutgeerts P. Omega-3 free fatty acids for the maintenance of remission in Crohn disease: the EPIC Randomized Controlled Trials. JAMA. 2008;299(14):1690-7.

Fiorenza AM, Branchi A, Sommariva D. Serum lipoprotein profile in patients with cancer. A comparison with non-cancer subjects. Int J Clin Lab Res. 2000;30(3):141-5.

Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. Gut microbes. 2012;3(4):289-306.

Garcia-Mazcorro JF, Lanerie DJ, Dowd SE, Paddock CG, Grutzner N, Steiner JM, Ivanek R, Suchodolski JS. Effect of a multi-species synbiotic formulation on fecal bacterial microbiota

of healthy cats and dogs as evaluated by pyrosequencing. FEMS Microbiol Ecol. 2011;78(3):542-54.

Garcia-Sancho M, Rodriguez-Franco F, Sainz A, Mancho C, Rodriguez A. Evaluation of clinical, macroscopic, and histopathologic response to treatment in nonhypoproteinemic dogs with lymphocytic-plasmacytic enteritis. J Vet Intern Med. 2007;21(1):11-7.

Garden OA, Pidduck H, Lakhani KH, Walker D, Wood JL, Batt RM. Inheritance of gluten-sensitive enteropathy in Irish Setters. Am J Vet Res. 2000;61(4):462-8.

Gaschen FP, Merchant SR. Adverse food reactions in dogs and cats. Vet Clin North Am Small Anim Pract. 2011;41(2):361-79.

Gauffin Cano P, Santacruz A, Moya A, Sanz Y. *Bacteroides uniformis CECT 7771* ameliorates metabolic and immunological dysfunction in mice with high-fat-diet induced obesity. PLoS ONE. 2012;7(7):e41079.

Gerbec Ž. Evaluation of therapeutic potential of restoring gastrointestinal homeostasis by a fecal microbiota transplant in dogs [master thesis]. Ljubljana: Univerza v Ljubljani; 2016.

German AJ, Hall EJ, Day MJ. Analysis of leucocyte subsets in the canine intestine. J Comp Pathol. 1999;120(2):129-45.

German AJ, Hall EJ, Day MJ. Chronic intestinal inflammation and intestinal disease in dogs. J Vet Intern Med. 2003a;17(1):8-20.

German AJ, Day MJ, Ruaux CG, Steiner JM, Williams DA, Hall EJ. Comparison of direct and indirect tests for small intestinal bacterial overgrowth and antibiotic-responsive diarrhea in dogs. J Vet Intern Med. 2003b;17(1):33-43.

Goldberg RJ, Katz J. A meta-analysis of the analgesic effects of omega-3 polyunsaturated fatty acid supplementation for inflammatory joint pain. Pain. 2007;129(1-2):210-23.

Goodwin LV, Goggs R, Chan DL, Allenspach K. Hypercoagulability in dogs with protein-losing enteropathy. J Vet Intern Med 2011; 25 (1): 273–277.

Goodwin S, McPherson JD, McCombie WR. Coming of age: ten years of next-generation sequencing technologies. Nat Rev Genet. 2016;17(6):333-51.

Gorzelak MA, Gill SK, Tasnim N, Ahmadi-Vand Z, Jay M, Gibson DL. Methods for improving human gut microbiome data by reducing variability through sample processing and storage of stool. PLoS ONE. 2015;10(8):e0134802.

Gough E, Shaikh H, Manges AR. Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent *Clostridium difficile* infection. Clin Infect Dis. 2011;53(10):994-1002.

Grützner N, Bishop MA, Suchodolski JS, Steiner JM. Association study of cobalamin deficiency in the Chinese Shar Pei. J Hered. 2010;101(2):211-7.

Guard BC, Barr JW, Reddivari L, Klemashevich C, Jayaraman A, Steiner JM, Vanamala J, Suchodolski JS. Characterization of microbial dysbiosis and metabolomic changes in dogs with acute diarrhea. PLoS ONE. 2015;10(5):e0127259.

Guard BC, Suchodolski JS. HORSE SPECIES SYMPOSIUM: Canine intestinal microbiology and metagenomics: From phylogeny to function. J Anim Sci. 2016;94(6):2247-61.

Gültekin M, Pasa S, Ural K, Balikci C, Ekren Aşici G, Gültekin G. Oxidative status and lipid profile among dogs at different stages of visceral Leishmaniasis. Türkiye Parazitol Derg. 2017;41:183-7.

Gundermann KJ, Kuenker A, Kuntz E, Drozdzik M. Activity of essential phospholipids (EPL) from soybean in liver diseases. Pharmacol Rep. 2011;63(3):643-59.

Gundermann KJ, Gundermann S, Drozdzik M, Mohan Prasad VG. Essential phospholipids in fatty liver: a scientific update. Clin Exp Gastroenterol. 2016;9:105-17.

Gupta S, Allen-Vercoe E, Petrof EO. Fecal microbiota transplantation: in perspective. Therap Adv Gastroenterol. 2016;9(2):229-39.

Hall EJ, German AJ. Diseases of the Small Intestine. In: Ettinger SJ, Feldmann EC, editors. Textbook of Veterinary Internal Medicine. 7th ed. St. Louis, Missouri, USA: Saunders Elsevier; 2010. p. 1526 -72.

Handl S, Dowd SE, Garcia-Mazcorro JF, Steiner JM, Suchodolski JS. Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. FEMS Microbiol Ecol. 2011;76(2):301-10.

Haucke V, Di Paolo G. Lipids and lipid modifications in the regulation of membrane traffic. Curr Opin Cell Biol. 2007;19(4):426-35.

He X, Huang Y, Li B, Gong CX, Schuchman EH. Deregulation of sphingolipid metabolism in Alzheimer's disease. Neurobiol Aging. 2010;31(3):398-408.

Heilmann RM, Grellet A, Allenspach K, Lecoindre P, Day MJ, Priestnall SL, Toresson L, Procoli F, Grützner N, Suchodolski JS, Steiner JM. Association between fecal S100A12 concentration and histologic, endoscopic, and clinical disease severity in dogs with idiopathic inflammatory bowel disease. Vet Immunol Immunopathol. 2014;158(3-4):156-66.

Heilmann RM, Volkmann M, Otoni CC, Grützner N, Kohn B, Jergens AE, Steiner JM. Fecal S100A12 concentration predicts a lack of response to treatment in dogs affected with chronic enteropathy. Vet J. 2016;215:96-100.

Heilmann RM, Allenspach K. Pattern-recognition receptors: signaling pathways and dysregulation in canine chronic enteropathies – brief review. J Vet Diagn Invest. 2017:1040638717728545.

Heilmann RM, Steiner JM. Clinical utility of currently available biomarkers in inflammatory enteropathies of dogs. J Vet Intern Med. 2018;32(5):1495-508.

Heilmann RM, Xenoulis PG, Müller K, Stavroulaki EM, Suchodolski JS, Steiner JM. Association of serum calprotectin (S100A8/A9) concentrations and idiopathic hyperlipidemia in Miniature Schnauzers. J Vet Intern Med. 2019;33(2):578-87.

Herstad KMV, Gajardo K, Bakke AM, Moe L, Ludvigsen J, Rudi K, Rud I, Sekelja M, Skancke E. A diet change from dry food to beef induces reversible changes on the faecal microbiota in healthy, adult client-owned dogs. BMC Vet Res. 2017;13(1):147.

Hess RS, Saunders HM, Van Winkle TJ, Shofer FS, Washabau RJ. Clinical, clinicopathologic, radiographic, and ultrasonographic abnormalities in dogs with fatal acute pancreatitis: 70 cases (1986-1995). J Am Vet Med Assoc. 1998;213(5):665-70.

Ho CS, Lam CW, Chan MH, Cheung RC, Law LK, Lit LC, Ng KF, Suen MW, Tai HL. Electrospray ionisation mass spectrometry: principles and clinical applications. Clin Biochem Rev. 2003;24(1):3-12.

Hong S, Gronert K, Devchand PR, Moussignac RL, Serhan CN. Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation. J Biol Chem. 2003;278(17):14677-87.

Honneffer JB, Minamoto Y, Suchodolski JS. Microbiota alterations in acute and chronic gastrointestinal inflammation of cats and dogs. World J Gastroenterol. 2014;20(44):16489-97.

Hossain Z, Hosokawa M, Takahashi K. Growth inhibition and induction of apoptosis of colon cancer cell lines by applying marine phospholipid. Nutr Cancer. 2008;61(1):123-30.

Jakobsson HE, Jernberg C, Andersson AF, Sjolund-Karlsson M, Jansson JK, Engstrand L. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. PLoS ONE. 2010;5(3):e9836.

Jantscheff P, Schlesinger M, Fritzsche J, Taylor LA, Graeser R, Kirfel G, Furst DO, Massing U, Bendas G. Lysophosphatidylcholine pretreatment reduces VLA-4 and P-Selectin-mediated b16.f10 melanoma cell adhesion in vitro and inhibits metastasis-like lung invasion in vivo. Mol Cancer Ther. 2011;10(1):186-97.

Jergens A, Moore F, Haynes J, Miles K. Idiopathic inflammatory bowel disease in dogs and cats: 84 cases (1987-1990). J Am Vet Med Assoc. 1992;201(10):1603-8.

Jergens AE, Schreiner CA, Frank DE, Niyo Y, Ahrens FE, Eckersall PD, Benson TJ, Evans R. A scoring index for disease activity in canine inflammatory bowel disease. J Vet Intern Med. 2003;17(3):291-7.

Jergens AE, Crandell J, Morrison JA, Deitz K, Pressel M, Ackermann M, Suchodolski JS, Steiner JM, Evans R. Comparison of oral prednisone and prednisone combined with metronidazole for induction therapy of canine inflammatory bowel disease: a randomized-controlled trial. J Vet Intern Med. 2010;24(2):269-77.

Jergens AE, Willard MD, Allenspach K. Maximizing the diagnostic utility of endoscopic biopsy in dogs and cats with gastrointestinal disease. Vet J. 2016;214:50-60.

Jeusette IC, Lhoest ET, Istasse LP, Diez MO. Influence of obesity on plasma lipid and lipoprotein concentrations in dogs. Am J Vet Res. 2005;66(1):81-6.

Jia J, Frantz N, Khoo C, Gibson GR, Rastall RA, McCartney AL. Investigation of the faecal microbiota associated with canine chronic diarrhoea. FEMS Microbiol Ecol. 2010;71(2):304-12.

Johnson MC. Hyperlipidemia disorders in dogs. Compend Contin Educ Vet. 2005;27:361-70.

Kassam Z, Lee CH, Yuan Y, Hunt RH. Fecal microbiota transplantation for Clostridium difficile infection: systematic review and meta-analysis. Am J Gastroenterol. 2013;108(4):500-8.

Kathrani A, House A, Catchpole B, Murphy A, German A, Werling D, Allenspach K. Polymorphisms in the TLR4 and TLR5 gene are significantly associated with inflammatory bowel disease in German shepherd dogs. PLoS ONE. 2010;5(12):e15740.

Kathrani A, House A, Catchpole B, Murphy A, Werling D, Allenspach K. Breed-independent toll-like receptor 5 polymorphisms show association with canine inflammatory bowel disease. Tissue Antigens. 2011;78(2):94-101.

Kathrani A, Holder A, Catchpole B, Alvarez L, Simpson K, Werling D, Allenspach K. TLR5 risk-associated haplotype for canine inflammatory bowel disease confers hyper-responsiveness to flagellin. PLoS ONE. 2012;7(1):e30117.

Kathrani A, Lee H, White C, Catchpole B, Murphy A, German A, Werling D, Allenspach K. Association between nucleotide oligomerisation domain two (Nod2) gene polymorphisms and canine inflammatory bowel disease. Vet Immunol Immunopathol. 2014;161(1-2):32-41.

Kelly CR, Kahn S, Kashyap P, Laine L, Rubin D, Atreja A, Moore T, Wu G. Update on fecal microbiota transplantation 2015: indications, methodologies, mechanisms, and outlook. Gastroenterology. 2015;149(1):223-37.

Kilpinen S, Spillmann T, Syrja P, Skrzypczak T, Louhelainen M, Westermarck E. Effect of tylosin on dogs with suspected tylosin-responsive diarrhea: a placebo-controlled, randomized, double-blinded, prospective clinical trial. Acta Vet Scand. 2011;53(1):26.

Kilpinen S, Spillmann T, Westermarck E. Efficacy of two low-dose oral tylosin regimens in controlling the relapse of diarrhea in dogs with tylosin-responsive diarrhea: a prospective, single-blinded, two-arm parallel, clinical field trial. Acta Vet Scand. 2014;56(1):43.

Kilpinen S, Rantala M, Spillmann T, Bjorkroth J, Westermarck E. Oral tylosin administration is associated with an increase of faecal enterococci and lactic acid bacteria in dogs with tylosin-responsive diarrhoea. Vet J. 2015;205(3):369-74.

Kimmel SE, Waddell LS, Michel KE. Hypomagnesemia and hypocalcemia associated with protein-losing enteropathy in Yorkshire terriers: five cases (1992–1998). J Am Vet Med Assoc. 2000;217(5):703-6.

Kluger EK, Malik R, Ilkin WJ, Snow D, Sullivan DR, Govendir M. Serum triglyceride concentration in dogs with epilepsy treated with phenobarbital or with phenobarbital and bromide. J Am Vet Med Assoc. 2008;233(8):1270-7.

Klymiuk I, Bambach I, Patra V, Trajanoski S, Wolf P. 16S based microbiome analysis from healthy subjects' skin swabs stored for different storage periods reveal phylum to genus level changes. Front Microbiol. 2016;7:2012.

Kolbjørnsen Ø, Press CM, Landsverk T. Gastropathies in the Lundehund. APMIS. 1994;102(7-12):647-61.

Kuehl F, Egan R. Prostaglandins, arachidonic acid, and inflammation. Science. 1980;210(4473):978-84.

Küllenberg D, Taylor LA, Schneider M, Massing U. Health effects of dietary phospholipids. Lipids in Health and Disease. 2012;11:3.

Lam SM, Shui G. Lipidomics as a principal tool for advancing biomedical research. J Genet Genomics. 2013;40(8):375-90.

Langer-Safer PR, Levine M, Ward DC. Immunological method for mapping genes on Drosophila polytene chromosomes. Proc Natl Acad Sci USA. 1982;79(14):4381-5.

Lenox CE, Bauer JE. Potential adverse effects of omega-3 fatty acids in dogs and cats. J Vet Intern Med. 2013;27(2):217-26.

Levy E, Rizwan Y, Thibault L, Lepage G, Brunet S, Bouthillier L, Seidman E. Altered lipid profile, lipoprotein composition, and oxidant and antioxidant status in pediatric Crohn disease. Am J Clin Nutr. 2000;71(3):807-15.

Li M, Fan P, Wang Y. Lipidomics in health and diseases – beyond the analysis of lipids. J Glycomics Lipidomics. 2014.

Li Q, Lauber CL, Czarnecki-Maulden G, Pan Y, Hannah SS. Effects of the dietary protein and carbohydrate ratio on gut microbiomes in dogs of different body conditions. mBio. 2017;8(1):e01703-16.

Lin PW, Myers LE, Ray L, Song SC, Nasr TR, Berardinelli AJ, Kundu K, Murthy N, Hansen JM, Neish AS. *Lactobacillus rhamnosus* blocks inflammatory signaling in vivo via reactive oxygen species generation. Free Radic Biol Med. 2009;47(8):1205-11.

Lindahl T. Instability and decay of the primary structure of DNA. Nature. 1993;362(6422):709-15.

Littman MP, Dambach DM, Vaden SL, Giger U. Familial protein-losing enteropathy and protein-losing nephropathy in Soft Coated Wheaten Terriers: 222 cases (1983-1997). J Vet Intern Med. 2000;14(1):68-80.

Liu L, Li Y, Li S, Hu N, He Y, Pong R, Lin D, Lu L, Law M. Comparison of next-generation sequencing systems. J Biomed Biotechnol. 2012;2012:251364.

Liu Q, Zerbinatti CV, Zhang J, Hoe HS, Wang B, Cole SL, Herz J, Muglia L, Bu G. Amyloid precursor protein regulates brain apolipoprotein E and cholesterol metabolism through lipoprotein receptor LRP1. Neuron. 2007;56(1):66-78.

Liu Q, Zhang J. Lipid metabolism in Alzheimer's disease. Neurosci Bull. 2014;30(2):331-45.

Lordan R, Tsoupras A, Zabetakis I. Phospholipids of animal and marine origin: structure, function, and anti-inflammatory properties. Molecules. 2017;22(11):1964.

Louis-Sylvestre J. Phosphatidylserine and memory problems in aged subjects. Cah Nutr Diet. 1999;34(6):349-57.

MacLachlan NJ, Breitschwerdt EB, Chambers JM, Argenzio RA, De Buysscher EV. Gastroenteritis of Basenji dogs. Vet Pathol. 1988;25(1):36-41.

Manchester AC, Hill S, Sabatino B, Armentano R, Carroll M, Kessler B, Miller M, Dogan B, McDonough SP, Simpson KW. Association between granulomatous colitis in French Bulldogs and invasive Escherichia coli and response to fluoroquinolone antimicrobials. J Vet Intern Med. 2013;27(1):56-61.

Mandigers PJ, Biourge V, van den Ingh TS, Ankringa N, German AJ. A randomized, open-label, positively-controlled field trial of a hydrolyzed protein diet in dogs with chronic small bowel enteropathy. J Vet Intern Med. 2010;24(6):1350-7.

Mansfield CS, James FE, Craven M, Davies DR, O'Hara AJ, Nicholls PK, Dogan B, MacDonough SP, Simpson KW. Remission of histiocytic ulcerative colitis in Boxer dogs correlates with eradication of invasive intramucosal *Escherichia coli*. J Vet Intern Med. 2009;23(5):964-9.

Marion-Letellier R, Savoye G, Beck PL, Panaccione R, Ghosh S. Polyunsaturated fatty acids in inflammatory bowel diseases: a reappraisal of effects and therapeutic approaches. Inflamm Bowel Dis. 2013;19(3):650-61.

Marks S, Laflamme DP, McAloose D. Dietary trial using a commercial hypoallergenic diet containing hydrolyzed protein for dogs with inflammatory bowel disease. Vet Ther. 2002;3:109-18.

Martín R, Miquel S, Chain F, Natividad JM, Jury J, Lu J, Sokol H, Theodorou V, Bercik P, Verdu EF, Langella P, Bermudez-Humaran LG. *Faecalibacterium prausnitzi*i prevents physiological damages in a chronic low-grade inflammation murine model. BMC Microbiol. 2015;15:67.

Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature. 2008;453(7195):620.

McMahon L, House A, Catchpole B, Elson-Riggins J, Riddle A, Smith K, Werling D, Burgener I, Allenspach K. Expression of Toll-like receptor 2 in duodenal biopsies from dogs with inflammatory bowel disease is associated with severity of disease. Vet Immunol Immunopathol. 2010;135(1):158-63.

Megalemou K, Sioriki E, Lordan R, Dermiki M, Nasopoulou C, Zabetakis I. Evaluation of sensory and in vitro anti-thrombotic properties of traditional Greek yogurts derived from different types of milk. Heliyon. 2017;3(1):e00227.

Mentula S, Harmoinen J, Heikkila M, Westermarck E, Rautio M, Huovinen P, Kononen E. Comparison between cultured small-intestinal and fecal microbiotas in beagle dogs. Appl Environ Microbiol. 2005;71(8):4169-75.

Middelbos IS, Vester Boler BM, Qu A, White BA, Swanson KS, Fahey GC, Jr. Phylogenetic characterization of fecal microbial communities of dogs fed diets with or without supplemental dietary fiber using 454 pyrosequencing. PLoS ONE. 2010;5(3):e9768.

Minamoto Y, Otoni CC, Steelman SM, Buyukleblebici O, Steiner JM, Jergens AE, Suchodolski JS. Alteration of the fecal microbiota and serum metabolite profiles in dogs with idiopathic inflammatory bowel disease. Gut microbes. 2015;6(1):33-47.

Miquel S, Martín R, Rossi O, Bermudez-Humaran LG, Chatel JM, Sokol H, Thomas M, Wells JM, Langella P. *Faecalibacterium prausnitzii* and human intestinal health. Curr Opin Microbiol. 2013;16(3):255-61.

Mori N, Lee P, Muranaka S, Sagara F, Takemitsu H, Nishiyama Y, Yamamoto I, Yagishita M, Arai T. Predisposition for primary hyperlipidemia in Miniature Schnauzers and Shetland sheepdogs as compared to other canine breeds. Res Vet Sci. 2010;88(3):394-9.

Mori N, Lee P, Kondo K, Kido T, Saito T, Arai T. Potential use of cholesterol lipoprotein profile to confirm obesity status in dogs. Vet Res Commun. 2011;35(4):223-35.

Mortier F, Strohmeyer K, Hartmann K, Unterer S. Acute haemorrhagic diarrhoea syndrome in dogs: 108 cases. Vet Rec. 2015;176(24):627.

Mueller RS, Fieseler KV, Fettman MJ, Zabel S, Rosychuk RA, Ogilvie GK, Greenwalt TL. Effect of omega-3 fatty acids on canine atopic dermatitis. J Small Anim Pract. 2004;45(6):293-7.

Müller MR, Linek M, Lowenstein C, Rothig A, Doucette K, Thorstensen K, Mueller RS. Evaluation of cyclosporine-sparing effects of polyunsaturated fatty acids in the treatment of canine atopic dermatitis. Vet J. 2016;210:77-81.

Murphy T, Chaitman J, Han E. Use of fecal transplant in eight dogs with refractory *Clostridium perfringens* associated diarrhea. J Vet Intern Med. 2014;28(3):976-1134.

Neish AS. Microbes in gastrointestinal health and disease. Gastroenterology. 2009;136(1):65-80.

Oelschlaeger TA. Mechanisms of probiotic actions – a review. Int J Med Microbiol. 2010;300(1):57-62.

Olivry T, DeBoer DJ, Favrot C, Jackson HA, Mueller RS, Nuttall T, Prelaud P. Treatment of canine atopic dermatitis: 2010 clinical practice guidelines from the International Task Force on Canine Atopic Dermatitis. Vet Dermatol. 2010;21(3):233-48.

Olthof MR, Brink EJ, Katan MB, Verhoef P. Choline supplemented as phosphatidylcholine decreases fasting and postmethionine-loading plasma homocysteine concentrations in healthy men. Am J Clin Nutr. 2005;82(1):111-7.

Ontsouka CE, Burgener IA, Mani O, Albrecht C. Polyunsaturated fatty acid-enriched diets used for the treatment of canine chronic enteropathies decrease the abundance of selected genes of cholesterol homeostasis. Domest Anim Endocrinol. 2010;38(1):32-7.

Ontsouka EC, Burgener IA, Luckschander-Zeller N, Blum JW, Albrecht C. Fish-meal diet enriched with omega-3 PUFA and treatment of canine chronic enteropathies. Eur J Lipid Sci Technol. 2012;114(4):412-22.

Packey CD, Sartor RB. Interplay of commensal and pathogenic bacteria, genetic mutations, and immunoregulatory defects in the pathogenesis of inflammatory bowel diseases. J Intern Med. 2008;263(6):597-606.

Pasquini A, Luchetti E, Cardini G. Plasma lipoprotein concentrations in the dog: the effects of gender, age, breed and diet. J Anim Physiol Anim Nutr (Berl). 2008;92(6):718-22.

Pavlidis P, Powell N, Vincent RP, Ehrlich D, Bjarnason I, Hayee B. Systematic review: bile acids and intestinal inflammation – luminal aggressors or regulators of mucosal defence? Aliment Pharmacol Ther. 2015;42(7):802-17.

Pereira GQ, Gomes LA, Santos IS, Alfieri AF, Weese JS, Costa MC. Fecal microbiota transplantation in puppies with canine parvovirus infection. J Vet Intern Med. 2018;32(2):707-11.

Pinna C, Vecchiato CG, Zaghini G, Grandi M, Nannoni E, Stefanelli C, Biagi G. In vitro influence of dietary protein and fructooligosaccharides on metabolism of canine fecal microbiota. BMC Vet Res. 2016;12:53.

Poongothai S, Karkuzhali K, Siva Prakash G, Sangaatha T, Saravanan T, Deepa R, Sharada G, Mohan V. Effect of essentiale in diabetic subjects with non-alcoholic fatty liver. Int J Diab Dev Countries. 2005;25(1):12-9.

Reis AC, Silva JO, Laranjeira BJ, Pinheiro AQ, Carvalho CB. Virulence factors and biofilm production by isolates of *Bacteroides fragilis* recovered from dog intestinal tracts. Braz J Microbiol. 2014;45(2):647-50.

Richter Y, Herzog Y, Lifshitz Y, Hayun R, Zchut S. The effect of soybean-derived phosphatidylserine on cognitive performance in elderly with subjective memory complaints: a pilot study. Clin Interv Aging. 2013;8:557-63.

Rioux KP, Madsen KL, Fedorak RN. The role of enteric microflora in inflammatory bowel disease: human and animal studies with probiotics and prebiotics. Gastroenterol Clin North Am. 2005;34(3):465-82, ix.

Ripolles Piquer B, Nazih H, Bourreille A, Segain JP, Huvelin JM, Galmiche JP, Bard JM. Altered lipid, apolipoprotein, and lipoprotein profiles in inflammatory bowel disease: consequences on the cholesterol efflux capacity of serum using Fu5AH cell system. Metabolism. 2006;55(7):980-8.

Romanato G, Scarpa M, Angriman I, Faggian D, Ruffolo C, Marin R, Zambon S, Basato S, Zanoni S, Filosa T, Pilon F, Manzato E. Plasma lipids and inflammation in active inflammatory bowel diseases. Aliment Pharmacol Ther. 2009;29(3):298-307.

Rossi G, Pengo G, Caldin M, Palumbo Piccionello A, Steiner JM, Cohen ND, Jergens AE, Suchodolski JS. Comparison of microbiological, histological, and immunomodulatory parameters in response to treatment with either combination therapy with prednisone and metronidazole or probiotic VSL#3 strains in dogs with idiopathic inflammatory bowel disease. PLoS ONE. 2014;9(4):e94699.

Sakakima Y, Hayakawa A, Nakao A. Phosphatidylcholine induces growth inhibition of hepatic cancer by apoptosis via death ligands. Hepatogastroenterology. 2009;56(90):481-4.

Sánchez E, De Palma G, Capilla A, Nova E, Pozo T, Castillejo G, Varea V, Marcos A, Garrote JA, Polanco I, Lopez A, Ribes-Koninckx C, Garcia-Novo MD, Calvo C, Ortigosa L, Palau F, Sanz Y. Influence of environmental and genetic factors linked to celiac disease risk on infant gut colonization by *Bacteroides* species. Appl Environ Microbiol. 2011;77(15):5316-23.

Sappati Biyyani RS, Putka BS, Mullen KD. Dyslipidemia and lipoprotein profiles in patients with inflammatory bowel disease. J Clin Lipidol. 2010;4(6):478-82.

Sauter SN, Allenspach K, Gaschen F, Grone A, Ontsouka E, Blum JW. Cytokine expression in an ex vivo culture system of duodenal samples from dogs with chronic enteropathies: modulation by probiotic bacteria. Domest Anim Endocrinol. 2005;29(4):605-22.

Sauter SN, Benyacoub J, Allenspach K, Gaschen F, Ontsouka E, Reuteler G, Cavadini C, Knorr R, Blum JW. Effects of probiotic bacteria in dogs with food responsive diarrhoea treated with an elimination diet. J Anim Physiol Anim Nutr (Berl). 2006;90(7-8):269-77.

Schmitz S, Glanemann B, Garden OA, Brooks H, Chang YM, Werling D, Allenspach K. A prospective, randomized, blinded, placebo-controlled pilot study on the effect of *Enterococcus faecium* on clinical activity and intestinal gene expression in canine food-responsive chronic enteropathy. J Vet Intern Med. 2015a;29(2):533-43.

Schmitz S, Werling D, Allenspach K. Effects of ex-vivo and in-vivo treatment with probiotics on the inflammasome in dogs with chronic enteropathy. PLoS ONE. 2015b;10(3):e0120779.

Schmitz S, Suchodolski J. Understanding the canine intestinal microbiota and its modification by pro-, pre- and synbiotics – what is the evidence? Vet Med Sci. 2016;2(2):71-94.

Schreiner NM, Gaschen F, Grone A, Sauter SN, Allenspach K. Clinical signs, histology, and CD3-positive cells before and after treatment of dogs with chronic enteropathies. J Vet Intern Med. 2008;22(5):1079-83.

Schroit AJ, Zwaal RFA. Transbilayer movement of phospholipids in red cell and platelet membranes. Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes. 1991;1071(3):313-29.

Scott KP, Gratz SW, Sheridan PO, Flint HJ, Duncan SH. The influence of diet on the gut microbiota. Pharmacol Res. 2013;69(1):52-60.

Seage EC, Drobatz KJ, Hess RS. Spectrophotometry and ultracentrifugation for measurement of plasma lipids in dogs with diabetes mellitus. J Vet Intern Med. 2018;32(1):93-8.

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12(6):R60.

Segata N, Boernigen D, Tickle TL, Morgan XC, Garrett WS, Huttenhower C. Computational meta'omics for microbial community studies. Mol Syst Biol. 2013;9(1):666.

Serhan CN, Hamberg M, Samuelsson B. Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human leukocytes. Proc Natl Acad Sci USA. 1984;81(17):5335-9.

Serhan CN, Clish CB, Brannon J, Colgan SP, Chiang N, Gronert K. Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2–nonsteroidal antiinflammatory drugs and transcellular processing. J Exp Med. 2000;192(8):1197-204.

Shores DR, Binion DG, Freeman BA, Baker PR. New insights into the role of fatty acids in the pathogenesis and resolution of inflammatory bowel disease. Inflamm Bowel Dis. 2011;17(10):2192-204.

Sim WH, Wagner J, Cameron DJ, Catto-Smith AG, Bishop RF, Kirkwood CD. Novel Burkholderiales 23S rRNA genes identified in ileal biopsy samples from children: preliminary evidence that a subtype is associated with perianal Crohn's disease. J Clin Microbiol. 2010;48(5):1939-42.

Simmerson SM, Armstrong PJ, Wunschmann A, Jessen CR, Crews LJ, Washabau RJ. Clinical features, intestinal histopathology, and outcome in protein-losing enteropathy in Yorkshire Terrier dogs. J Vet Intern Med. 2014;28(2):331-7.

Simopoulos AP. Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr. 2002;21(6):495-505.

Simpson KW, Jergens AE. Pitfalls and progress in the diagnosis and management of canine inflammatory bowel disease. Vet Clin North Am Small Anim Pract. 2011;41(2):381-98.

Sinha R, Abnet CC, White O, Knight R, Huttenhower C. The microbiome quality control project: baseline study design and future directions. Genome Biol. 2015;16:276.

Slovak JE, Wang C, Sun Y, Otoni C, Morrison J, Deitz K, LeVine D, Jergens AE. Development and validation of an endoscopic activity score for canine inflammatory bowel disease. Vet J. 2015;203(3):290-5.

Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottiere HM, Dore J, Marteau P, Seksik P, Langella P. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A. 2008;105(43):16731-6.

Stokes JE, Kruger JM, Mullaney T, Holan K, Schall W. Histiocytic ulcerative colitis in three non-boxer dogs. J Am Anim Hosp Assoc. 2001;37(5):461-5.

Suchodolski JS, Ruaux CG, Steiner JM, Fetz K, Williams DA. Assessment of the qualitative variation in bacterial microflora among compartments of the intestinal tract of dogs by use of a molecular fingerprinting technique. Am J Vet Res. 2005;66(9):1556-62.

Suchodolski JS, Camacho J, Steiner JM. Analysis of bacterial diversity in the canine duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis. FEMS Microbiol Ecol. 2008;66(3):567-78.

Suchodolski JS, Dowd SE, Westermarck E, Steiner JM, Wolcott RD, Spillmann T, Harmoinen JA. The effect of the macrolide antibiotic tylosin on microbial diversity in the canine small intestine as demonstrated by massive parallel 16S rRNA gene sequencing. BMC Microbiol. 2009;9(1):210.

Suchodolski JS, Xenoulis PG, Paddock CG, Steiner JM, Jergens AE. Molecular analysis of the bacterial microbiota in duodenal biopsies from dogs with idiopathic inflammatory bowel disease. Vet Microbiol. 2010;142(3-4):394-400.

Suchodolski JS, Dowd SE, Wilke V, Steiner JM, Jergens AE. 16S rRNA gene pyrosequencing reveals bacterial dysbiosis in the duodenum of dogs with idiopathic inflammatory bowel disease. PLoS ONE. 2012a;7(6):e39333.

Suchodolski JS, Markel ME, Garcia-Mazcorro JF, Unterer S, Heilmann RM, Dowd SE, Kachroo P, Ivanov I, Minamoto Y, Dillman EM, Steiner JM, Cook AK, Toresson L. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. PLoS ONE. 2012b;7(12):e51907.

Suchodolski JS. Diagnosis and interpretation of intestinal dysbiosis in dogs and cats. Vet J. 2016;215:30-7.

Swanson KS, Dowd SE, Suchodolski JS, Middelbos IS, Vester BM, Barry KA, Nelson KE, Torralba M, Henrissat B, Coutinho PM, Cann IK, White BA, Fahey GC, Jr. Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. ISME J. 2011;5(4):639-49.

Tamai R, Furuya M, Hatoya S, Akiyoshi H, Yamamoto R, Komori Y, Yokoi S, Tani K, Hirano Y, Komori M, Takenaka S. Profiling of serum metabolites in canine lymphoma using gas chromatography mass spectrometry. J Vet Med Sci. 2014;76(11):1513-8.

Taylor LA, Pletschen L, Arends J, Unger C, Massing U. Marine phospholipid – a promising new dietary approach to tumor-associated weight loss. Support Care Cancer. 2010;18(2):159.

Titmarsh H, Gow AG, Kilpatrick S, Sinclair J, Hill T, Milne E, Philbey A, Berry J, Handel I, Mellanby RJ. Association of vitamin D status and clinical outcome in dogs with a chronic enteropathy. J Vet Intern Med. 2015;29(6):1473-8.

Touvier M, Fassier P, His M, Norat T, Chan DS, Blacher J, Hercberg S, Galan P, Druesne-Pecollo N, Latino-Martel P. Cholesterol and breast cancer risk: a systematic review and meta-analysis of prospective studies. Br J Nutr. 2015;114(3):347-57.

Tsorotioti SE, Nasopoulou C, Detopoulou M, Sioriki E, Demopoulos CA, Zabetakis I. In vitro anti-atherogenic properties of traditional Greek cheese lipid fractions. Dairy Sci Technol. 2014;94(3):269-81.

Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett C, Knight R, Gordon JI. The human microbiome project: exploring the microbial part of ourselves in a changing world. Nature. 2007;449(7164):804-10.

Ungaro F, Rubbino F, Danese S, D'Alessio S. Actors and factors in the resolution of intestinal inflammation: Lipid mediators as a new approach to therapy in inflammatory bowel diseases. Front Immunol. 2017;8:1331.

Unterer S, Busch K, Leipig M, Hermanns W, Wolf G, Straubinger RK, Mueller RS, Hartmann K. Endoscopically visualized lesions, histologic findings, and bacterial invasion in the gastrointestinal mucosa of dogs with acute hemorrhagic diarrhea syndrome. J Vet Intern Med. 2014;28(1):52-8.

Vaden SL, Hammerberg B, Davenport DJ, Orton SM, Trogdon MM, Melgarejo LT, VanCamp SD, Williams DA. Food hypersensitivity reactions in Soft Coated Wheaten Terriers with protein-losing enteropathy or protein-losing nephropathy or both: gastroscopic food sensitivity testing, dietary provocation, and fecal immunoglobulin E. J Vet Intern Med. 2000;14(1):60-7.

van der Meer-Janssen YP, van Galen J, Batenburg JJ, Helms JB. Lipids in host-pathogen interactions: pathogens exploit the complexity of the host cell lipidome. Prog Lipid Res. 2010;49(1):1-26.

van Eijk M, Aten J, Bijl N, Ottenhoff R, van Roomen CP, Dubbelhuis PF, Seeman I, Ghauharali-van der Vlugt K, Overkleeft HS, Arbeeny C, Groen AK, Aerts JM. Reducing glycosphingolipid content in adipose tissue of obese mice restores insulin sensitivity, adipogenesis and reduces inflammation. PLoS ONE. 2009;4(3):e4723.

van Meer G. Cellular lipidomics. EMBO J. 2005;24(18):3159-65.

Vázquez-Baeza Y, Hyde ER, Suchodolski JS, Knight R. Dog and human inflammatory bowel disease rely on overlapping yet distinct dysbiosis networks. Nat Microbiol. 2016;1:16177.

Videlock EJ, Cremonini F. Meta-analysis: probiotics in antibiotic-associated diarrhoea. Aliment Pharmacol Ther. 2012;35(12):1355-69.

Vitale CL, Olby NJ. Neurologic dysfunction in hypothyroid, hyperlipidemic Labrador Retrievers. J Vet Intern Med. 2007;21(6):1316-22.

Viviano KR. Update on immununosuppressive therapies for dogs and cats. Vet Clin North Am Small Anim Pract. 2013;43(5):1149-70.

Vogeser M, Parhofer KG. Liquid chromatography tandem-mass spectrometry (LC-MS/MS) – technique and applications in endocrinology. Exp Clin Endocrinol Diabetes. 2007;115(9):559-70.

Vrieze A, Van Nood E, Holleman F, Salojarvi J, Kootte RS, Bartelsman JF, Dallinga-Thie GM, Ackermans MT, Serlie MJ, Oozeer R, Derrien M, Druesne A, Van Hylckama Vlieg JE,

Bloks VW, Groen AK, Heilig HG, Zoetendal EG, Stroes ES, de Vos WM, Hoekstra JB, Nieuwdorp M. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology. 2012;143(4):913-6.e7.

Wagner Mackenzie B, Waite DW, Taylor MW. Evaluating variation in human gut microbiota profiles due to DNA extraction method and inter-subject differences. Front Microbiol. 2015;6:130.

Washabau RJ, Day MJ, Willard MD, Hall EJ, Jergens AE, Mansell J, Minami T, Bilzer TW. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. J Vet Intern Med. 2010;24(1):10-26.

Watson P, Simpson KW, Bedford PG. Hypercholesterolaemia in briards in the United Kingdom. Res Vet Sci. 1993;54(1):80-5.

Weese J, Costa M, Webb J. Preliminary clinical and microbiome assessment of stool transplantation in the dog and cat. J Vet Intern Med. 2013;27(3):604-756.

Wenk MR. The emerging field of lipidomics. Nat Rev Drug Discov. 2005;4(7):594-610.

Wenk MR. Lipidomics: new tools and applications. Cell. 2010;143(6):888-95.

Westermarck E, Skrzypczak T, Harmoinen J, Steiner JM, Ruaux CG, Williams DA, Eerola E, Sundbäck P, Rinkinen M. Tylosin-responsive chronic diarrhea in dogs. J Vet Intern Med. 2005;19(2):177-86.

Wexler HM. *Bacteroides*: the good, the bad, and the nitty-gritty. Clin Microbiol Rev. 2007;20(4):593-621.

Whittemore JC, Suchodolski JS. The role of probiotics in the management of intestinal dysbiosis. people. 2016;3:5.

Wright-Rodgers AS, Waldron MK, Bigley KE, Lees GE, Bauer JE. Dietary fatty acids alter plasma lipids and lipoprotein distributions in dogs during gestation, lactation, and the perinatal period. J Nutr. 2005;135(9):2230-5.

Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature. 2007;448(7152):427-34.

Xenoulis PG, Suchodolski JS, Levinski MD, Steiner JM. Investigation of hypertriglyceridemia in healthy Miniature Schnauzers. J Vet Intern Med. 2007;21(6):1224-30.

Xenoulis PG, Palculict B, Allenspach K, Steiner JM, Van House AM, Suchodolski JS. Molecular-phylogenetic characterization of microbial communities imbalances in the small intestine of dogs with inflammatory bowel disease. FEMS Microbiol Ecol. 2008;66(3):579-89.

Xenoulis PG, Steiner JM. Lipid metabolism and hyperlipidemia in dogs. Vet J. 2010;183(1):12-21.

Xenoulis PG, Suchodolski JS, Ruaux CG, Steiner JM. Association between serum triglyceride and canine pancreatic lipase immunoreactivity concentrations in Miniature Schnauzers. J Am Anim Hosp Assoc. 2010;46(4):229-34.

Xenoulis PG, Steiner JM. Canine hyperlipidaemia. J Small Anim Pract. 2015;56(10):595-605.

Xu J, Verbrugghe A, Lourenco M, Janssens GP, Liu DJ, Van de Wiele T, Eeckhaut V, Van Immerseel F, Van de Maele I, Niu Y, Bosch G, Junius G, Wuyts B, Hesta M. Does canine

inflammatory bowel disease influence gut microbial profile and host metabolism? BMC Vet Res. 2016;12(1):114.

Yamashita A, Hayashi Y, Nemoto-Sasaki Y, Ito M, Oka S, Tanikawa T, Waku K, Sugiura T. Acyltransferases and transacylases that determine the fatty acid composition of glycerolipids and the metabolism of bioactive lipid mediators in mammalian cells and model organisms. Prog Lipid Res. 2014;53:18-81.

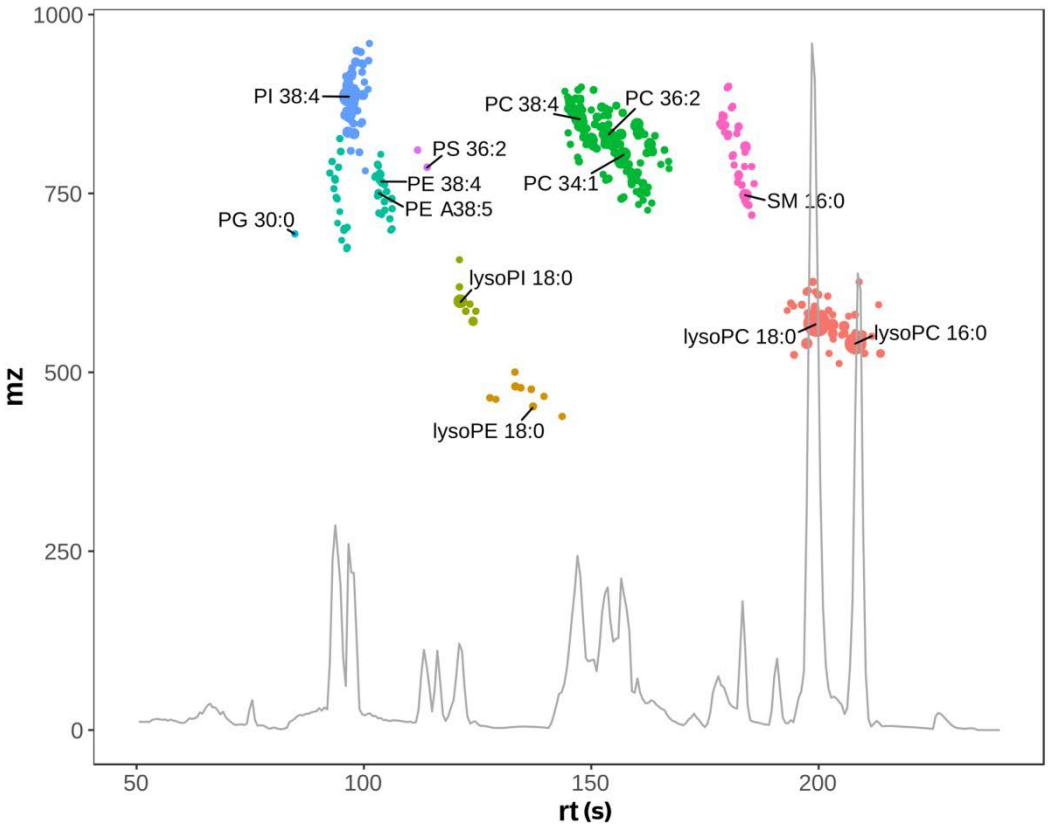
Yilmaz Z, Senturk S. Characterisation of lipid profiles in dogs with parvoviral enteritis. J Small Anim Pract. 2007;48(11):643-50.

Zarfoss MK, Dubielzig RR. Solid intraocular xanthogranuloma in three Miniature Schnauzer dogs. Vet Ophthalmol. 2007;10(5):304-7.

Zicker SC, Jewell DE, Yamka RM, Milgram NW. Evaluation of cognitive learning, memory, psychomotor, immunologic, and retinal functions in healthy puppies fed foods fortified with docosahexaenoic acid-rich fish oil from 8 to 52 weeks of age. J Am Vet Med Assoc. 2012;241(5):583-94.

8 APPENDIX

Supporting Information of Publication "Comparison of the Systemic Phospholipid Profile in Dogs Diagnosed with Idiopathic Inflammatory Bowel Disease or Food-Responsive Diarrhea before and after Treatment" (see chapter 3.2)



S1 Fig. Base peak chromatogram of one of the samples. Base peak chromatogram of the separation by hydrophilic interaction liquid chromatography of phospholipids extracted from blood of a dog with FRD before treatment. Colored dots indicate retention time and m/z ratio of phospholipids detected by orbitrap ultrahigh resolution mass spectrometry.
https://doi.org/10.1371/journal.pone.0215435.s001

S2 Fig. Effect of treatment on the individual phospholipids. The dashed red horizontal line is located at p = 0.05, with dots above the line having p-values < 0.05; p values < 0.05 considered as significant.
https://doi.org/10.1371/journal.pone.0215435.s002

S1 Table. Characteristics of the dogs with IBD (n=16) or FRD (n=16) included in the study.

Group characteristic	IBD	FRD	*P*-value
Total number	16	16	-
Age in years, median (IQR)	4.8 (3.1–7.4)	2.5 (1.2–6.1)	**0.020***
Sex, male/female	9/7	8/8	0.723**
Body weight in kg, median (IQR)	21.8 (9.2–32.4)	24.3 (10.7–33.9)	0.665*
Body condition score, median (IQR)	5 (4–6)	5 (4–6)	0.638*
Breed, n (%)			
▪ Pure-bred dogs	11 (69%)	12 (75%)	0.694**
▪ Mixed breed dogs	5 (31%)	4 (25%)	
CIBDAI score, median (IQR)	7 (4–12)	5 (4–8)	0.201*

∗*P*-values obtained by Wilcoxon rank-sum test. *P*-values < 0.05 considered as significant.
∗∗*P*-values obtained by likelihood ratio test for association. *P*-values < 0.05 considered as significant.
https://doi.org/10.1371/journal.pone.0215435.s003

S2 Table. Raw peaklist of annotated phospholipids. Raw peaklist including the numbers of all 331 phospholipid signals in the samples analyzed. Phospholipids were annotated based on retention time and mass to charge (m/z) ratio. Phospholipids annotated with an '*' had a difference between theoretical and observed m/z of > 0.015 Da (but < 0.050) and should be considered 'tentatively identified'. Retention times and observed m/z values are included in the peaklist. Disease category: red = IBD; blue = IBD with PLE; green = FRD.
https://doi.org/10.1371/journal.pone.0215435.s004

S3 Table. Sample identification and patient information.
https://doi.org/10.1371/journal.pone.0215435.s005

S4 Table. P-values of effect of treatment, disease category and sample type on individual phospholipids.
https://doi.org/10.1371/journal.pone.0215435.s006

S1 File. Nutritional composition of the study diet.

Adult Sensitive Gastrointestinal
Codfish and Rice *

AVERAGE ANALYSIS	
Moisture	8 %
Crude protein	24 %
Crude fat	13 %
Crude ash	6.5 %
Crude fiber	2.5 %
Carbohydrates (NfE)	45 %
Dietary fibers	6 %
Starch	

MINERALS	
Calcium	1.1 %
Phosphorus	0.8 %
Potassium	0.54 %
Sodium	0.4 %
Magnesium	0.1 %
Iron	125 mg/kg
Copper	15 mg/kg
Manganese	20 mg/kg
Zinc	200 mg/kg
Iodine	2 mg/kg
Selenium	200 mcg/kg

VITAMINS	
Vitamin A	13000 UI
Vitamin D3	1500 UI
Vitamin E	100 mg
Vitamin B1	10 mg
Vitamin B2	20 mg
Pantothenic acid	30 mg
Niacin (PP)	40 mg
Vitamin B6	8 mg
Vitamin B12	100 mcg
Biotin	0.8 mg
Folic acid	2 mg
Choline	1900 mg
Biocholine	225 mg

FATTY ACIDS	
Fatty acids (Omega 6)	3.5 %
Fatty acids (Omega 3)	1.0 %

ENERGY	/kg
Metabolizable E. (Atwater)	3939 kcal
Metabolizable E. (AAFCO)	3520 kcal
Metabolizable E. (NCR)	3680 kcal

SPEC.	
L-Carnitine	100 mg/kg
DL-Methionine	330 mg/kg

Biomill Adult Sensitive Gastrointestinal is a quality product made in Switzerland, developed in cooperation with the University of Bern and distributed by Biomill AG, 1523 Granges-Marnand (Switzerland) www.biomill.ch FREECALL 0800 554 310 only in Switzerland

22.08.08 DIs

S2 File. Table of nutritional content (original in French).

A L L I X ²

Edition Recettes en colonne

BIOMIL

Groupe		Blum		Dossier	Fab
Liste de Prix		2008 net		Jeu	totales

	Etab. : **Formule :** **N° Ordre :** **N° Optim :**		**05** **347** **5031** **8571**	**05** **890** **5154** **8070**	**05** **891** **5155** **8054**
	Prix de la Recette		92.90	114.39	122.80
0001	POIDS	100	100.00	100.00	100.00
0002	MS	%	88.71	90.50	90.50
0003	PROTEINE	%	23.74	26.65	26.65
0004	GRAISSE	%	13.01	16.51	16.51
0004	C18 2 n6	%	2.72	3.86	3.12
0004	C18 3 n3	%	0.58	0.74	1.37
0005	CELL	%	2.13	2.12	2.12
0005	FIBRES	%	6.31	6.74	6.74
0006	MIN cendres	%	6.37	7.77	7.77
0007	NfE	%	43.46	37.45	37.45
0007	GE	KJ/	1816.50	1919.11	1919.11
0007	sV GE	%	88.46	88.48	88.48
0008	Energy NCR	KJ/	1516.51	1600.09	1600.09
0009	Energie AAFCO	KJ/	1447.21	1526.36	1526.36
0009	Energie AAFCO	CA	345.75	364.65	364.65
0011	LYS	%	1.48	1.87	1.87
0012	MET	%	0.48	0.65	0.65
0013	CYS	%	0.44	0.43	0.43
0014	M+C	%	0.93	1.10	1.10
0015	THR	%	1.00	1.17	1.17
0016	TRP	%	0.25	0.28	0.28
0017	ARGIN	%	1.22	1.41	1.41
0018	HISTID	%	0.53	0.61	0.61
0019	ISOLEU	%	1.11	1.29	1.29
0020	CARNITI	Gr	0.10	0.10	0.10
0029	CA	%	1.09	1.28	1.28
0030	PHOS	%	0.82	0.92	0.92
0031	PHOD	%	0.74	0.75	0.75
0032	K	%	0.52	0.58	0.58
0033	NA	%	0.41	0.43	0.43
0034	CL	%	0.66	0.70	0.70
0035	MAGNE	%	0.10	0.11	0.11
0036	FER	Gr	0.10	0.10	0.10
0037	CU	Gr	0.01	0.01	0.01
0038	MANGAN	Gr	0.02	0.02	0.02
0039	ZINC	Gr	0.20	0.20	0.20
0040	IODE	Gr	0.00	0.00	0.00
0041	SELENIUM	mg	0.20	0.20	0.20
0042	COBALT	mg	0.12	0.11	0.11
0039	ZINC org.	Gr	0.05	0.05	0.05
0045	VIT. A	Kui	13.40	13.40	13.40
0046	VIT. D3	Kui	1.50	1.50	1.50
0047	VIT. E	Gr	0.10	0.10	0.10
0050	VIT. B1	Gr	0.01	0.01	0.01
0051	VIT. B2	Gr	0.02	0.02	0.02
0052	PANTOTH A	Gr	0.03	0.03	0.03
0053	NIACIN B3	Gr	0.04	0.04	0.04
0054	PYRIDOX B6	Gr	0.01	0.01	0.01
0055	FOLIQUE A	Gr	0.00	0.00	0.00
0056	VIT. B12	mg	0.10	0.10	0.10
0057	BIOTINE	mg	0.80	0.76	0.76
0058	CHOLINE	Gr	1.98	2.78	2.78
0058	BiochoLIN	Gr	0.22	0.22	0.22
0061	amidon	%	35.89	27.95	27.95

S3 File. Results of external PUFA analysis by Swiss reference laboratory (original in French).

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UFAG LABORATORIEN
Untersuchung und Forschung

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Liste de distribution: Moulins de Granges SA, Mme Valérie Vincent, Granges-Marnand
Facture: Moulins de Granges SA, Granges-Marnand

Rapport

Rapport No.: 07-23945

Reçu le: 24.12.07 Terminé le: 10.01.08

Sommaire

Pos.	Numéro d'éch.	Nom, Désignation
1	07-23945-001	Diet low (Art. 89C)

Sursee, 10.01.2008

Votre interlocuteur (-trice):
Susanne Täuber
Leiterin Analytik Lebensmittel

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Rapport

Rapport No.: 07-23945

Reçu le: 24.12.07 Terminé le: 10.01.08

Client: Moulins de Granges SA, KdNr. 15490
Mme Valérie Vincent
CH-1523 Granges-Marnand

Position: 1
Numéro d'éch.: 07-23945-001
Nom: Diet low (Art. 89C)
Note: Prod.: 20.12.2007

Méthode, technique de mesure Objet d'analyse		Résultat	Unité	Valeur réf.	Valeur tol.	Valeur limite	LDT / LD
Nutritives							
MSDA; gravimétrique (hydr. acide)							
Matières grasses		16.9	g/100g				
Acides gras							
MSDA; GC-FID							
A.caproique	C 6	ndt	g/100g				0.1
A.caprylique	C 8	ndt	g/100g				0.1
A.caprique	C 10	ndt	g/100g				0.1
Acide laurique	C 12	ndt	g/100g				0.1
Acide myristique	C 14	0.2	g/100g				
Acide palmitique	C 16	3.2	g/100g				
Acide palmitoléique	C 16:1	0.5	g/100g				
Acide margarique	C 17	ndt	g/100g				0.1
Acide stéarique	C 18	0.9	g/100g				
Acide oléique	C 18:1	5.4	g/100g				
Acide linolique	C 18:2	3.9	g/100g				
alpha-acide linolénique	C 18:3	0.4	g/100g				
gamma-acide linolénique	C 18:3	ndt	g/100g				0.1
A.arachidique	C 20	ndt	g/100g				0.1
Acide gadoléique	C 20:1	0.1	g/100g				
A.eicosadienoï	C 20:2	ndt	g/100g				0.1
A.eicosatrién.	C 20:3	ndt	g/100g				0.1
A.arachidonique	C 20:4	0.1	g/100g				
A.eicosapentaé	C 20:5	0.2	g/100g				
A.béhénique	C 22	ndt	g/100g				0.1
Acide erucique	C 22:1	ndt	g/100g				0.1
A.docosadiénoi.	C 22:2	ndt	g/100g				0.1
A.docosatétraé.	C 22:4	ndt	g/100g				0.1
A.docosapentaé.	C 22:5	0.1	g/100g				
A.docosahexaé.	C 22:6	0.3	g/100g				
Acide lignocérique	C 24	ndt	g/100g				0.1
Acide sélacholéique	C 24:1	ndt	g/100g				0.1
Acide octadécatétr.	C 18:4	ndt	g/100g				0.1
Acides gras totales							
Acide gras saturés		4.3	g/100g				
Acides gras mono-insat.		6.0	g/100g				

Légende: nd = non détectable (inférieure à la LD) LD = Limite de détection UFC = Unités formant colonie
ndt = non déterminable (inférieure à la LI LDT = Limite de détermination MS = Matière sèche

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Position: 1
Numéro d'éch.: 07-23945-001
Nom: Diet low (Art. 89C)

Objet d'analyse	Résultat	Unité	Valeur réf.	Valeur tol.	Valeur limite	LDT / LD
Méthode, technique de mesure						
Acides gras totales						
MSDA; GC-FID						
Acides gras polyinsat.	5.0	g/100g				
Ac. gras polyins. oméga-3	1.0	g/100g				
Ac. gras polyins. oméga-6	4.0	g/100g				

Légende: nd = non détectable (inférieure à la LD) LD = Limite de détection UFC = Unités formant colonie
ndt = non déterminable (inférieure à la LI LDT = Limite de détermination MS = Matière sèche

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9 ACKNOWLEDGMENTS

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